8-2021

Utilization of Protective Cultures to Reduce Late Gas Formation by Paucilactobacillus wasatchensis

Ireland R. Green
Utah State University

UTILIZATION OF PROTECTIVE CULTURES TO REDUCE LATE GAS FORMATION BY *Paucilactobacillus wasatchensis*

by

Ireland R. Green

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

Approved:

_____	_____
Donald J. McMahon, Ph. D. Major Professor	Craig J. Oberg, Ph.D. Committee Member
_____	_____
Jeffrey R, Broadbent, Ph.D. Committee Member	Randall K. Thunell, Ph.D. Committee Member

_____
D. Richard Cutler, Ph.D.
Interim Vice Provost for Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2021

ABSTRACT

Utilization of Protective Cultures to Reduce Late Gas Formation by *Paucilactobacillus wasatchensis*

by

Ireland Green, Master of Science

Utah State University, 2021

Major Professor: Dr. Donald J. McMahon
Department: Nutrition, Dietetics and Food Sciences

Paucilactobacillus wasatchensis is a non-starter lactic acid bacterium that causes late gas formation defect in aged Cheddar cheese. This results in slits and cracks, which make the cheese difficult to shred or slice and less appealing to consumers. When *Plb. wasatchensis* grows to high numbers in cheese, it can produce carbon dioxide as it metabolizes galactose or other six-carbon compounds in the cheese. A risk factor for such gas formation is use of *Streptococcus thermophilus* as part of the starter culture to speed up acid production during cheesemaking. Using higher temperatures to accelerate aging is another risk factor. The aim of this research was to find adjunct cultures (protective cultures) that can deplete galactose in cheese but not interfere with cheesemaking by fermenting lactose and then determine the effect of these cultures on reducing gas formation by *Plb. wasatchensis.*

From 40 potential cultures screened, 3 cultures had these characteristics. A model system to determine gas production was developed using carbohydrate-restricted MRS broth containing galactose and ribose that, when inoculated with 10^5 CFU/mL of *Plb.*

wasatchensis, always produced gas. Three protective cultures were inoculated at 10^3 to 10^7 CFU/mL into this model system using two sets of tubes (in triplicate). One test tube was used to measure optical density and sampled to measure galactose and bacterial numbers. Galactose concentration was determined enzymatically, and bacteria enumerated on MRS+ribose agar plates. The other tube contained an inverted Durham tube resting on a capillary tube to monitor gas production. In the test tubes containing only *Plb. wasatchensis,* gas was observed on d 8 with galactose levels gradually dropping during incubation to 0.15% by 8 d, and completely gone by 12 d. When any of the protective cultures were added (even at only 10^3 CFU/mL initially) to tubes that also contained *Plb. wasatchensis,* galactose was depleted by d 4 with no gas bubbles observed by 12 d. This shows the potential for using protective adjunct cultures to remove residual galactose from cheese to reduce unwanted slits and cracks.

(70 Pages)

PUBLIC ABSTRACT

Utilization of Protective Cultures to Reduce Late Gas Formation by *Paucilactobacillus wasatchensis*

Ireland Green

Cheesemaking is a process susceptible to defects caused by microbes not purposefully added by the manufacturer. While these bacteria are not a health concern, they can cause problems such as producing carbon dioxide inside the cheese. This can cause slits and cracks in cheese making cheese unsuitable for slicing. One such bacteria identified by cheese researchers at Utah State University and Weber State University is *Paucilactobacillus wasatchensis.* When present in cheese, it can cause gas formation by using galactose to supply its energy. In doing so, it takes this six-carbon sugar and converts it into a five-carbon sugar and at the same time releasing carbon dioxide.

The research team proposed a two-year project to develop a model system for studying this unwanted gas formation. This also involved investigation of other bacteria that could be added which use up these sugars and prevent *Plb. wasatchensis* from producing the unwanted gas. The BUILD Dairy program of the Western Dairy Center provided $85,000 for graduate student support and laboratory expenses for this project as a way to increase student investigations to solve problems faced by the dairy industry.

Information on the successful use of protective cultures to metabolize galactose before *Lb. wasatchensis* could use it to make carbon dioxide has been disseminated through presentations at regional and national microbiology, dairy, and cheese industry conferences.

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to Dr. Donald J. McMahon, my advisor, for giving me the opportunity do this project under his mentorship at Utah State University. His many hours of guidance, support, and patience are what made this project possible. I would also like to extend my deepest gratitude to Dr. Craig J. Oberg, Dr. Jeff Broadbent, and Dr. Randall Thunell for their expertise and mentorship. The substantial amount of time and consideration the above-mentioned professors were willing to put into this project and attempting to make me a better scientist means more to me than I could ever put into words. I greatly appreciate the support received from Dr. Michele Culumber, Dr. Matthew Domek, Dr. Michael Lefevre, Dr. Almut Vollmer, Dr. Taylor Oberg, and Dr. Prateek Sharma. I would also like to thank the BUILD Dairy Program for funding this project and giving me opportunities to be involved in the Dairy industry. Finally, I would like to thank my family, especially my incredible son, for the many sacrifices they made to ensure my success.

Ireland Green

CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

SLAB = Starter lactic acid bacteria

NSLAB = Non-starter lactic acid bacteria

WDC04 = type strain of *Paucilactobacillus wasatchensis*

LAB = Lactic acid bacteria

GRAS = Generally Recognized as Safe

FDA = Food and Drug Administration

CFU/mL = Colony forming units/milliliter

MRS = de Man, Rogosa, and Sharpe media

BCP = Bromocresol purple

MRS+R = de Man, Rogosa, and Sharpe media supplemented with 1% ribose

MRS+G = de Man, Rogosa, and Sharpe media supplemented with 1% galactose

OD = OD_{600} - Optical Density at 600 nm

CR-MRS = carbohydrate restricted de Man, Rogosa, and Sharpe media

GPB = Gas Production Broth

GPT = Gas Production Test

INTRODUCTION

Cheddar cheese harbors a complex microbiota of lactic acid bacteria (**LAB**) along with other bacteria, yeasts, and molds which, although studied for many years, continues to perplex both members of the dairy industry and food scientists. In bacterial ripened cheeses like Cheddar, the LAB can be separated into two distinct groups: starter LAB (**SLAB**) which are bacteria purposely added by the producer during the cheesemaking process and non-starter LAB (**NSLAB**), which are not intentionally added and initially present at low cell numbers compared to SLAB. The NSLAB enter the cheese because they either survive pasteurization or are introduced post-pasteurization from the dairy plant environment.

Some NSLAB are viewed as a positive or desired addition to the cheese microbiota while others are associated with defects during the Cheddar cheese ripening such as slits and cracks, off-flavors, and crystal formation (Crow et al., 1995; Broadbent et. al., 2003). *Paucilactobacillus* wasatchensis, formerly designated as *Lactobacillus wasatchensis* (Oberg et al., 2016), and presumptively referred to as *Lactobacillus wasatchii* (Ortakci et al., 2015a) is a gram positive, rod shaped, obligatory heterofermentative NSLAB shown to cause late gas formation in aging Cheddar cheese (Ortakci et al., 2015b). The type strain for this species is *Plb. wasatchensis* WDC04 (Oberg et al., 2016). The ability of *Plb. wasatchensis* WDC04 to utilize galactose by the heterofermentative phosphoketolase pathway results in CO_2 production when its preferential carbohydrate source (ribose) is limiting (Ortakci et al., 2015a).

One strategy for preventing unwanted gas formation in cheese is to add another LAB strain during cheesemaking to remove any residual galactose in cheese.

Manufacturers could use this culture as an adjunct in Cheddar cheese to control late gas defect. Growth of *Plb. wasatchensis* when galactose is the only available sugar is slow since it preferentially utilizes ribose but it can slowly co-utilize galactose when ribose is also present (Ortakci et al., 2015a). Utilization of galactose by *Plb. wasatchensis* after growing to high numbers in the cheese can result in late gas defect because of CO_2 production during galactose metabolism. Microbial interference in such gas production could be achieved by using a LAB strain that ferments galactose (**Gal**$^+$), but not lactose (**Lac**$^-$), to remove the substrate needed for gas production by *Plb. wasatchensis* without interfering with the cheese making process. Such preferential utilization of galactose by selected adjunct LAB early in cheese ripening period would inhibit gas production by *Plb. wasatchensis.* This research will develop a model system to test for gas production by *Plb. wasatchensis* and demonstrate the dependence of galactose availability on such gas production.

HYPOTHESIS AND OBJECTIVES

Hypothesis. Utilization of galactose by a Lac^- Gal^+ adjunct LAB when grown in combination with *Plb. wasatchensis* would prevent gas production by *Plb. wasatchensis.*

***Objectives*.**

1. Screen potential NSLAB adjunct cultures for those that are Gal^+ but Lac^-.
2. Develop a model gas production test (**GPT**) to reproducibly measure growth and CO_2 production by *Plb. wasatchensis* WDC04 by using different ratios of ribose and galactose, and different inoculum concentrations.
3. Determine the ratio of *Plb. wasatchensis* WDC04 to Lac^- Gal^+ LAB needed to prevent gas formation in the model gas production system.
4. Measure the galactose concentration present during incubation of *Plb. wasatchensis* WDC04 with the Lac^- Gal^+ LAB in the model gas production system.

LITERATURE REVIEW

Cheddar Cheese Manufacture

Cheddar cheese is a semi-hard variety of bacterial-ripened cheese generally aged at low temperatures over a course of 3 to 12 months. Traditionally, the steps for Cheddar cheese manufacture consist of coagulating milk (already inoculated with SLAB) using rennet, cutting coagulum into cubes, stirring and heating until the desired curd pH is reached, removing whey, cheddaring (pushing curd particles together to create knit curd by stacking curd blocks), cutting the curd, salting, pressing, packaging, and finally, aging (Papademas and Bintisis, 2017). Each step of the process is important for the final product to have the characteristic Cheddar taste, texture, look, and mouth feel. While the basic steps of Cheddar cheese manufacture remain similar, a few notable changes have occurred with advancing technology. Mechanized systems have been utilized to allow control of amount of moisture lost at any given time and pH-controlled production of starter cultures makes acid production more consistent and SLAB are more salt tolerant (Lawrence et al., 1984; McMahon et al., 2014). Specifically, these mechanized steps allow for more control of make times, product consistency, and finished cheese chemistry. This is important in large-scale automated plants because steps in the process have less ability to be modified than in small-scale artisan cheesemaking.

Lactic Acid Bacteria

Milk is an ideal growth media for microorganisms due to its almost neutral pH, high water content, and available nutrients (Montville and Matthews, 2005). However, milk is generally pasteurized before cheesemaking to reduce its microbial load and kill pathogens. The microbiology of Cheddar cheese plays an important role in the

manufacture of this cheese. Lactic acid bacteria are the main group of microorganisms used in manufacture of dairy products including primarily species of *Lactococcus, Lactobacillus, Streptococcus* and *Leuconostoc.* These are all catalase-negative, non-motile, non-spore forming, gram positive rods or cocci that produce lactic acid as the major end product of glucose fermentation (Orsi and Zambrini, 2017). The primary role of LAB in fermented dairy products such as Cheddar cheese is to convert lactose to lactic acid and, consequently, lower pH of the product. When LAB are added for manufacture of fermented dairy products, they can be categorized as SLAB (added to produce lactic acid) and adjunct LAB (i.e., those added for other reasons such as flavor development). The NSLAB are either already in the milk from the natural microbiota when non-pasteurized milk is used or from environmental contamination post pasteurization.

Starter Lactic Acid Bacteria

Starter cultures used in cheese making are classified into either single or mixed stains, and defined or undefined-strain starters. These have become much more reproducible in recent years through the use of pH-control during their manufacture, making them more consistent when used for cheese making. Manufacture of bacterial ripened cheese requires the use of SLAB due to their contribution to a favorable end product by: fermenting sugars (lowers pH, inhibits pathogenic bacteria growth), protein hydrolysis (involved in taste and texture development), synthesis of flavor compounds and texturing agents, and production of inhibitory compounds (Orsi and Zambrini, 2017).

Starter culture activity (metabolism) helps create the redox potential, moisture level, and pH in the cheese curd desired for the development of Cheddar flavor (Lowrie et al., 1974). Traditionally, parameters such as temperature and salt-in-moisture needed to

be monitored during manufacture to ensure metabolic ability of starter culture remained low after the cheese was manufactured (Lawrence et al., 1984). By the end of cheesemaking, there should be enough intact metabolically active cells to hydrolyze any remaining lactose and produce an anaerobic environment inside the cheese block (Crow et al., 1995).

Starter cultures can be used by the cheese manufacturer as bulk cultures or direct-to-vat set cultures. Bulk cultures are those in which the cheese manufacturer purchases a frozen stock culture from a commercial supplier and then prepares a large volume of liquid culture in their own facility. Aliquots of this bulk culture are then added to each cheese vat. Bulk cultures require stringent aseptic technique as they are prone to contamination. Direct-to-vat set culture preparation consists of purchasing and storing sufficient quantities of individual aliquots of frozen or lyophilized culture that are added directly to the milk in the cheese vat. This helps reduce the likelihood of contamination of starter culture by utilizing preservation methods such as deep-freezing and concentration methods (Orsi and Zambrini, 2017).

Traditionally, Cheddar cheese manufacture is defined by the use of *Lactococcus lactis* as the starter culture and a low cook temperature (39^{o}C) (Michel and Martley, 2001). Alternatively, a "short method" for the make of Cheddar cheese can be utilized to increase the rate of lactic acid production, thus decreasing the make time, through the use of a thermophilic lactic acid bacteria, *Streptococcus thermophilus,* in addition to the *Lc. lactis* in the starter culture (Bley et al., 1985; Michel and Martley, 2001). Initially, this was proposed so as to use a higher cook temperature of 42 to 43^{o}C by using *S. thermophilus* as the main starter bacteria along with *Lc. lactis* (Bley et al., 1985). More

commonly today, the cook temperature is not increased, but the presence of the *S. thermophilus* maintains faster acid production after cooking rather than the slowdown that occurs when *Lc. lactis* is used exclusively.

Utilization of *S. thermophilus* also provides some benefit due to its phage unrelatedness to *Lc. lactis* phages (Michel and Martley, 2001). However, the majority of *S. thermophilus* strains are unable to utilize galactose, which causes this sugar to accumulate in the cheese. Tinson et al. (1982) observed Cheddar cheese made with *Lc. lactis* subsp. *cremoris* starter and *S. thermophilus* adjunct cultures resulted in high levels of residual galactose (0.56% wt/wt) and high production of CO_2. Ortakci et al. (2015c), added *Plb. wasatchensis* WDC04 to Cheddar cheese made with *S. thermophilus* as the starter culture and also observed increased levels of gas formation at an elevated ripening temperature (12^oC).

While many LAB can ferment lactose, this carbohydrate is usually depleted in cheese by the time NSLAB populations grow to high numbers. This means NSLAB must look for other carbohydrates to use as energy sources (Williams et al, 2000). The pathway used by some LAB to metabolize lactose and, sometimes, glucose tends to leave large amounts of galactose which accumulates in fermented dairy products. This accumulation of galactose occurs because many strains aren't able to utilize the Tagatose-6P or Leloir pathways properly (Wu et al., 2015). Surplus galactose in Cheddar cheese made with *S. thermophilus* provides a ready substrate for NSLAB, including *Plb. wasatchensis.*

Non-starter Lactic Acid Bacteria

Milk used for Cheddar cheese making is usually pasteurized; however, some NSLAB already present in the raw milk may survive this treatment or become damaged

but recover (Jordan and Cogan, 1993; Somers et al., 2001). Nonstarter lactic acid bacteria may also be introduced into the milk post-pasteurization from the air, or from biofilms on dairy plant equipment, pipes, and surfaces. The NSLAB population is primarily comprised of mesophilic *Lactobacillus* and *Pediococcus* species (Jordan and Cogan, 1993; Crow et al., 1995). While initial numbers of NSLAB tend to be relatively low in freshly made cheese ($<10^2$ colony forming units (**CFU**)/g), they increase steadily during the aging process (after lactose has been depleted) and often become the predominate bacteria by the time the Cheddar is ready to be sold (Oberg et al., 2011; McMahon et al., 2014). Some NSLAB have positive effects on the cheese quality, but others have been associated with defects during ripening including slits and cracks, off-flavors, and crystal formation (Crow et al., 2001; Ortakci et al., 2015b, 2015c). Nonetheless, it is widely believed that good aged Cheddar cheese relies on both the SLAB and NSLAB cultures to create the taste, texture, and look of the product which pleases both the manufacturer and the customer (Orsi and Zambrini, 2017).

A defect of great concern in the Cheddar cheese industry is late gas formation. The resulting slits and cracks, along with gas expanded packaging, created by this defect cause reduced consumer satisfaction and these slits/cracks cause difficulty in cutting and slicing the cheese, which results in economic losses to the manufacturer. This defect in Cheddar cheese has historically been associated with a NSLAB due to its appearance later in the aging process when the NSLAB population increases in number, but the specific LAB genera and species responsible has been difficult to identify.

Paucilactobacillus wasatchensis is a gram positive, rod shaped, obligatory heterofermentative NSLAB (Oberg et al., 2016) determined to cause late gas formation in

Cheddar cheese (Ortakci et al., 2015b). While it was determined that the type strain, *Plb. wasatchensis* WDC04, preferentially utilizes ribose, the ability of this microorganism to slowly metabolize galactose and other 6-carbon carbohydrates is the concern in cheese. When galactose fermentation occurs, a carbon molecule is cleaved off leaving a 5-carbon sugar to be metabolized with the cleaved carbon molecule incorporated into CO_2 (Ortakci et al., 2015a and b).

Adjunct Cultures

Adjuncts are added to the milk during cheesemaking to improve product consistency, increase flavor intensity or provide unique flavor profiles (Fox et al., 1998). For Cheddar cheese, these bacteria are often isolated from the NSLAB populations of good quality cheese, and have also been utilized to inhibit other NSLAB (protective cultures), produce probiotic cheeses, accelerate ripening, improve aroma, and reduce bitterness (Wilkinson, 1993; Oberg et al., 2011).

Adjunct LAB cultures, like other LAB commonly found in human food, typically fall under the category of generally recognized as safe (**GRAS**) which is a term given by the United States Food and Drug Association (**FDA**) under sections 201(s) and 409 of the Federal Food, Drug, and Cosmetics Act (FDA, 2018). This status is granted in three basic ways: (1) if the chemical or product was in use before 1958 and has been confirmed safe based on common use (requires substantial history of safe consumption for food use by a large population of consumers), (2) the FDA grants the additive the title due to industry request, or (3) a company is able to provide enough evidence of safety through research. Manufacturers desire to have their adjunct cultures have GRAS status because then they don’t have to go through a premarket review or FDA approval before the additive or

organism can be put in their product to be sold, saving them significant time and money. Consumers like to see GRAS additives in their food rather than synthetic compounds because they are considered organic, extracted from “natural” sources, and generally don’t have intimidating, long names (Montville and Matthews, 2005).

Protective adjunct cultures are added to cheese along with the starter culture, where they compete with or inhibit growth of undesirable microorganisms through their metabolic activity in a manner that prevents or controls growth or end-product formation by undesired microorganisms in the food. The higher the cell number of protective cultures in terms of colony forming units present in milk (CFU/mL) or cheese (CFU/gm), the higher their effectiveness tends to be. However, adding higher numbers of adjunct bacteria initially, increases the cost of the intervention and could affect food quality (Orsi and Zambrini, 2017).

PRELIMINARY RESEARCH

Forty LAB cultures (Table 1) comprised mostly pediococci and lactobacilli, were obtained from LAB collections at Utah State University (Logan, UT), Weber State University (Ogden, UT) and Vivolac Inc. (Greenfield, IN). Each culture was screened for purity, gas formation ability, and utilization of galactose (Gal+) but inability to ferment lactose (Lac^-). To determine purity, De Man, Rogosa, and Sharp (**MRS;** Becton, Dickinson and Company, Franklin Lakes, NJ) agar plates were inoculated using a quadrant streak method then incubated anaerobically using Gaspak EZ anaerobic system (Becton, Dickinson and Company). Purity was assumed when only one type of colony size and morphology was observed for each culture.

Lactic acid bacterial cultures were incubated at their optimum growth temperature for 48 h. Individual cultures were then screened for sugar utilization using Bromocresol Purple (**BCP**) agar (Becton, Dickinson and Company) plates supplemented with either 1% galactose or 1% lactose. Then M-17 broth (Becton, Dickinson and Company) supplemented with 1% galactose and BCP broth supplemented with 1% of either galactose or lactose in test tubes containing inverted Durham tubes (Fisher Scientific, Waltham, MA) were then inoculated and incubated at the specific culture's optimum growth temperature for 48 h to determine gas formation and confirm sugar metabolism capabilities of each culture. Once specific LAB cultures were found that were Gal^+ but Lac^-, did not produce gas, and appeared pure, their overall carbohydrate fermentation ability was determined using API50 CH panels (BioMeriex, Marcy-l'Etoile, France).

Table 1. Cultures tested to determine Lac^{-} Gal^{+} metabolic ability.

Culture Number	2020 Genus[1]	Former Designation	Strain
1	*Lacticaseibacillus*	*Lactobacillus rhamnosus*	ATCC 27773
2	-[2]	*Lactobacillus delbrueckii*	ATCC 11842
3	-	*Lactobacillus delbrueckii* subsp. *bulgaricus*	ATCC 7995
4	*Lacticaseibacillus*	*Lactobacillus casei*	UW4
5		*Lactobacillus helveticus*	LH100
6	*Lacticaseibacillus*	*Lactobacillus rhamnosus*	ATCC 14957
8	*Lacticaseibacillus*	*Lactobacillus casei*	M36
9	-	*Lactobacillus helveticus*	LH32
10	*Lactiplantibacillus*	*Lactobacillus plantarum*	25F
11	*Lacticaseibacillus*	*Lactobacillus casei*	37
12	-	*Lactobacillus helveticus*	ATCC 7995
13	*Lacticaseibacillus*	*Lactobacillus paracasei*	Lila
14	-	*Bifidobacterium lactis*	DSM B-94
15	*Lacticaseibacillus*	*Lactobacillus rhamnosus*	ATCC 7469
16	*Lacticaseibacillus*	*Lactobacillus casei*	431
17	*Lacticaseibacillus*	*Lactobacillus casei* subsp. *pseudoplantarum*	ATCC 25598
18	-	*Lactobacillus acidophilus*	Neb
19	*Levilactobacillus*	*Lactobacillus brevis*	ATCC 367

21	*Lacticaseibacillus*	*Lactobacillus casei*	F19
23	-	*Streptococcus thermophilus*	TA061
24	*Lacticaseibacillus*	*Lactobacillus casei*	ATCC 393
25	*Lacticaseibacillus*	*Lactobacillus casei*	PN 5474
26	*Lacticaseibacillus*	*Lactobacillus casei*	PN 5547
27	*Lacticaseibacillus*	*Lactobacillus casei*	PN 5078
29	*Lacticaseibacillus*	*Lactobacillus casei*	CT
30	*Lacticaseibacillus*	*Lactobacillus casei*	LC10
31	*Lactiplantibacillus*	*Lactobacillus plantarum*	ATCC 367
32	*Lactilactobacillus*	*Lactobacillus curvatus*	WSU1
33	*Levilactobacillus*	*Lactobacillus brevis*	ATCC 13698
34	-	*Pediococcus* species	PaF
35	-	*Streptococcus thermophilus*	Hansen 104-405
36	-	*Pediococcus pentosaceus*	FBB61
37	-	*Pediococcus pentosaceus*	SPL-1
38	-	*Pediococcus pentosaceus*	SPL-2
39	-	*Pediococcus acidilactici*	23F
40	-	*Pediococcus pentosaceus*	LMB 11847

[1]Change in taxonomic nomenclature (Zheng et al., 2020)
[2]No change in nomenclature.

Thirty-four of the cultures did not meet the selection criteria, specifically exhibiting galactose fermentation (Gal^+) while not fermenting lactose (Lac^-). Six cultures met the preliminary screening parameters and were selected for additional characterization as part of the experimental protocol to be confirmed as candidates for use as protective adjunct cultures; *Lacticaseibacillus casei* UW4, *Lactobacillus helveticus* 7995, *Lacticaseibacillus casei* subsp. *pseudoplantarum* 25598, *Levilactobacillus brevis* 367, *Pediococcus pentosaceus* FBB61 and *Pediococcus acidilactici* 23F.

MATERIALS AND METHODS

Cultures

Frozen cultures (stored at -80°C) of the cultures previously determined to be Lac^- and Gal^+ using BCP broth were *Lcb. casei* UW4, *Lb. helveticus* 7995, *Lcb. casei* subsp. *pseudoplantarum* 25598, *Lvlb. brevis* 367, *Pc. pentosaceus* FBB61 and *Pc. acidilactici* 23F. These cultures were propagated in MRS broth and incubated at their individual optimum growth temperatures (20 to 35°C) for 24 h. *Paucilactobacillus wasatchensis* WDC04 was obtained from Weber State University and grown in MRS broth with 1% ribose (Sigma Aldrich Inc., St. Louis, MO) (**MRS+R**) and incubated at 25°C for 48-72 h. Multiple freezer tubes of each culture were stored at -80°C in preparation for subsequent experiments.

Culture Confirmation

Protective Cultures. For the previously identified Lac^- Gal^+ cultures, 16S rRNA sequencing was performed to confirm identity. Each culture was propagated overnight in MRS broth then a Masterpure gram positive DNA purification kit (Lucigen, Middleton, WI) was used to isolate and purify the DNA. A set of primers designed for use with LAB referred to as UF1 (5'-AGAGTTTGATCCTGGCTCAG-3') and UR1 (5'-GCTGGCACGTAGTTAGCC-3') was used (Broadbent et al., 2003). This set of forward and reverse primers amplify 520 bp from the 5' end of the 16S rRNA gene using PCR. If the 16S rRNA sequencing came back inconclusive, a second pair of universal primers UF2 (5'-GCACAAGSGGTGGAC-3' and UR2 (5'-TTGTCACCGGCAGTCT-3') were used that amplified a distal 235 bp DNA segment from the 5' end of the 16S rRNA gene using PCR (Broadbent et al., 2003).

The solutions used for the PCR reaction were: 10 µL of GoTaq green reaction buffer (Promega, Sunnyvale, CA), 1 µL of each of the forward and reverse primers, 1 µL of dNTP mic (Promega), 32.8 µL of nuclease-free water (Qiagen, Venlo, Netherlands), 0.5 µL of GoTaq DNA polymerase (Promega), and 4 µL of the DNA extract. The PCR reaction was then carried out using a DNA Thermal Cycler (PerkinElmer, Waltham, MA). Forty PCR cycles were performed using the following guidelines: 2-min soak at 94°C, followed by 30 cycles of 15 s at 94°C, 1 min at 55°C, and 1.5 min at 72°C . The reaction was finished by incubation at 72°C until samples were removed and stored at °°C until sequencing (Broadbent et al., 2003).

To confirm amplicons, 15 µL of each amplicon were loaded into the well of a 1.5% agarose gel. A DNA ladder (Hi-Lo DNA marker, Bionexus, Oakland, CA) was run in the gel for band reference. Electrophoresis was carried out in a Bio-Tad Wide Mini-Sub Cell (Bio-Rad, Hercules, Ca) at 80V for 45 min. The gel was stained with ethidium bromide (0.5 µg/mL) and photographed under UV light in a MultiDoc-It Digital Imaging System (UVP, Upland, CA). Confirmed amplicons were sent to the Center for Integrated Biosystems (Utah State University, Logan, UT) for sequencing. The resulting 16S rRNA sequences were analyzed using a nucleotide BLAST search (NCBI, Bethesda, MD) for identification.

Paucilactobacillus wasatchensis. A rapid detection method for *Plb. wasatchensis* WDC04 was used to confirm identity. The culture was propagated for 48 h in MRS+R broth. A Masterpure gram positive DNA purification kit (Lucigen, Middleton, WI) was used to isolate and purify the DNA. The primer set 86F (CTTGCACCAGATTGAGAGAACATT) and 258R

(TCCATCCAAAAGTGATAGCACAAG) was used due to the primer set's high specificity for *Plb. wasatchensis* (Culumber et. al., 2017). The PCR reaction and electrophoresis were then carried out in the same manner as the protective cultures.

Growth Curves

Paucilactobacillus wasatchensis. A standard growth curve for *Plb. wasatchensis* WDC04 was determined in MRS+R broth (pH 6.5). Sixteen-millimeter test tubes containing 10 mL of media were inoculated with 0.2 mL frozen stock solution of *Plb. wasatchensis* WDC04 in triplicate. Optical density (**OD_{600}**) was measured every 12 h using a spectrophotometer (Spectronic 200; Fisher Scientific, Fair Lawn, NJ). Plate counts were performed from serial dilutions in sterile 9 ml DW dilution blanks using 0.1 mL spread onto MRS+R agar plates and incubated at 23°C for 48 h in a Gaspak EZ system (Becton Dickinson, Sparks, MD). A calibration was then made between OD_{600} and CFU/mL so *Plb. wasatchensis* WDC04 solutions containing 10^9 CFU/mL could be prepared for further experiments.

Protective Cultures. Protective cultures were grown in MRS broth with 1% galactose added (MRS+G) for 18 h with the OD_{600} measured each hour as well as samples for plate counts collected aseptically. Plate counts were performed from serial dilutions inoculated onto MRS+G agar and incubated anaerobically at 30°C for 48 h using Gaspak EZ system. For studies in which protective cultures were combined with *Plb. wasatchensis* WDC04, the protective cultures were grown in the same manner and then adjusted with sterile MRS+G to the OD_{600} that, on a pooled average, delivered 10^9 CFU/ml. The maximum growth rate (μ_{max}) was calculated as the steepest protion of the growth curves.

Development of a Model System to Determine Gas Production

***Paucilactobacillus wasatchensis* WDC04.** To prepare broth solutions containing specified concentrations of selected substrates, a carbohydrate-restricted MRS (**CR-MRS**) broth was prepared using the following amounts per liter (Ortakci et al., 2015c): 10.0 g protease peptone No. 3 (EMD Chemicals Inc., Gibbstown, NJ), 10.0 g beef extract (Becton Dickinson, Sparks, MD), 5.0 g yeast extract (Becton Dickinson), 5.0 g sodium acetate (Sigma Aldrich Co., St. Louis, MO), 2.0 g ammonium citrate (Sigma Aldrich Co.), 2.0 g dipotassium phosphate (Fisher Scientific, Fair Lawn, NJ), 1.0 g Tween-80 (Sigma Aldrich Co.), 0.1 g magnesium sulfate (Fisher Scientific), and 0.05 g manganese sulfate (Avantor Performance Materials, Inc., Center Valley, PA). Stock solutions of galactose (Sigma Aldrich Inc., St. Louis, MO) and ribose at a 10% concentration (wt/vol) were filter sterilized (Filter 430768, Corning Inc. Corning, NY) and stored at 22°C . Different ratios of galactose and ribose were added to the CR-MRS broth for a total of 1% (wt/vol) carbohydrate. The ribose:galactose ratios used were: 0:100, 50:50, 40:60, 30:70, 20:80 and 10:90. Each sugar ratio broth solution was inoculated in two sets of triplicate tubes (6 tubes total) with *Plb. wasatchensis* WDC04 at the following inoculum levels 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 CFU/mL. In one set of test tubes, a Durham tube (Fisher Scientific) was inverted onto a 60-mm capillary tube (Drummond Scientific Company, Broomall, PA) at the bottom of the test tube prior to being sterilized so as to observe gas production. The second set of tubes in each replicate were used to measure changes in OD_{600}.

Following the first set of gas production trials using various ratios of ribose and galactose along with various inoculum concentrations for *Plb. wasatchensis* WDC04,

those combinations of both parameters that showed consistent gas production were selected for a further trial. Five additional replicates of the most consistent gas-producing sugar ratios and inoculum levels were run using the experimental protocol previously described to determine the optimum sugar ratio/inoculum level. The ribose:galactose ratio and *Plb. wasatchensis* WDC04 inoculum level required to always produce enough CO_2 to observe a gas bubble in the Durham was then determined. The optimum sugar ratio was designated as the Gas Production Broth (**GPB**) and the GPB along with the *Plb. wasatchensis* WDC04 inoculum concentration was then designated as the GPT.

Effect of Protective Cultures on Gas Production

Protective Cultures. Gas production test tubes were prepared containing GPB (0.3% ribose:0.7% galactose) in CR-MRS with 10^5 CFU/mL *Plb. wasatchensis* WDC04 were then inoculated with 10^3, 10^4, 10^5, 10^6, and 10^7 CFU/mL of each protective culture. Two sets of inoculated tubes were incubated at 25°C with gas production and OD_{600} measured as previously described. GPB control tubes were inoculated with *Plb. wasatchensis* WDC04, but not with a protective culture. The experiment was done in triplicate.

Galactose Analysis

Galactose was measured using the Megazyme Lactose and D-Galactose Assay Procedure (Megazyme, Bray, Ireland). A standard curve was developed using 99% galactose (Acros Organics, New Jersey, USA) added to CR-MRS media at levels of 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 0.75% (wt/vol) in duplicate tubes. From each varying actual percentage of galactose added to MRS-CR broth, the percentage of galactose was calculated according to the ΔAD-galactose = [(A2-A1)galactose sample - (A2-

A1)galactose blank] * dilution factor formula suggested in the Megazyme kit. The R^2 value (0.9991) obtained from the standard curve showed that the actual and calculated percentage of galactose values were nearly identical, therefore, the formula mentioned above was used for all further galactose percentage determinations from the absorbance values resulting from use of the Megazyme galactose kit. Instructions for the assay were followed using appropriate 20-fold dilutions so the galactose concentration was in the range of 4 to 80 μg in the 3-mL cuvette. Galactose was then measured in the CFR-MRS media containing either the protective cultures, *Plb. wasatchensis* WDC04, or for various combinations of protective cultures and *Plb. wasatchensis* WDC04 as mentioned in the previous section. Galactose concentration in the broth was calculated as [(A2-A1) of test sample – (A2-A1) of blank] multiplied by the dilution factor.

Statistical Analysis

Growth curves of *Plb. wasatchensis* WDC04 with accompanying gas production measurements at various ribose:galactose concentrations were performed in triplicate. An additional 5 replicates were performed for the combinations of ribose:galactose ratio and inoculum level to determine the most consistent conditions needed for gas production and for selection of optimum GPB parameters. Protective culture growth curves and inhibition of gas production by *Plb. wasatchensis* WDC04 were performed in triplicate. Protective culture inhibition of *Plb. wasatchensis* WDC04 controls and the Megazyme galactose assay standard curve were done in duplicate. Differences in growth curves as a result of adding a protective culture were analyzed using a random-effects model. The remaining experiments were conducted using a nested factorial design. Statistical

analysis was performed using Tukey HSD in JMP (version 14; SAS Statistical Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Protective Culture Selection

Carbohydrate fermentation based upon API CHL50 varied among the six cultures (Table 2). All cultures could ferment glucose but had differing capabilities of fermenting other carbohydrates. Fermentation of ribose and N-acetylglucosamine occurred with all cultures except *Lb. helveticus* 7995 and this may influence their ability to obtain energy for growth and metabolism during storage of cheese. *Pc. pentosaceous* FBB61 was observed in preliminary trials using BCP broth to not utilize lactose but tested positive for lactose utilization using APICH 50 and was eliminated from further testing.

When a species confirmation of the Lac^- Gal^+ cultures was performed using 16S rRNA sequencing, two cultures, *Lcb. casei* subsp. *pseudoplantarum* ATCC 25598 and *Lpb. plantarum* ATCC 367 were both determined to actually be *Pc. acidilactici* species. The other four cultures were correctly speciated. To avoid unnecessary duplication of species *Pc. acidilactici* 23F was selected while the other two *Pc. acidilactici* stains, ATCC 25598 and ATCC 367, were removed from further testing.

Growth Curves

***Paucilactobacillus wasatchensis* WDC04.** When grown in MRS+R broth at 23°C there was no increase in OD_{600} was observed until ~30 h followed by an exponential increase over the next ~30 h (Figure 1). However, based upon plate count numbers, no definitive lag phase was apparent and exponential growth was observed for the first 60 h followed by flattening in the growth curve indicating the culture entered the stationary phase. This was anticipated as they had been conditioned to grow in MRS+R media prior to inoculation.

Table 2. Ability of Lac$^-$ Gal$^+$ cultures (Lactocaseibacillus casei UW4, Lactobacillus helveticus 7995, Lactocaseibacillus. casei subsp. pseudoplantarum 25598, Lactiplantibacillus plantarum 367, Pediococcus pentosaceous FBB61 and Pediococcus acidilactici 23F) to ferment carbohydrates[1] based upon API50 CH testing.

Carbohydrate	Culture					
	UW4	7995	25598	367	FBB61	23F
L-Arabinose	-[3]	-	+[2]	+	+	+
D-Ribose	+	-	+	+	+	+
D-Xylose	-	-	+	+	-	+
D-Galactose	**+**	**+**	**+**	**+**	**+**	**+**
D-Glucose	+	+	+	+	+	+
D-Fructose	+	-	+	+	+	+
D-Mannose	+	-	+	-	+	+
D-Rhamnose	-	-	+	-	-	-
Dulcitol	-	+	-	-	-	-
D-Mannitol	+	-	-	+	-	-
Methyl α-D-glucopyranoside	-	-	-	+	-	-
N-Acetylglucosamine	+	-	+	+	+	+
Amygdalin	-	-	-	-	+	+
Arbutin	+	-	+	-	+	+
Esculin	+	-	+	+	+	+
Salicin	+	-	+	-	+	+
D-Cellobiose	+	-	+	-	+	+
D-Maltose	+	-	-	+	+	-
Lactose	**-**	**-**	**-**	**-**	**+**	**-**
D-Melibiose	-	-	-	+	-	-
D-Saccharose	-	-	-	-	-	+
D-Trehalose	+	-	+	-	+	+
D-Melezitose	+	-	-	-	-	-
Gentiobiose	+	-	+	-	+	+

[1]Carbohydrates for which no color change was observed by any cultures are not included.
[2]Bromocresol Purple indicator turned from purple to yellow (or to black for esculin)
[3]No color change

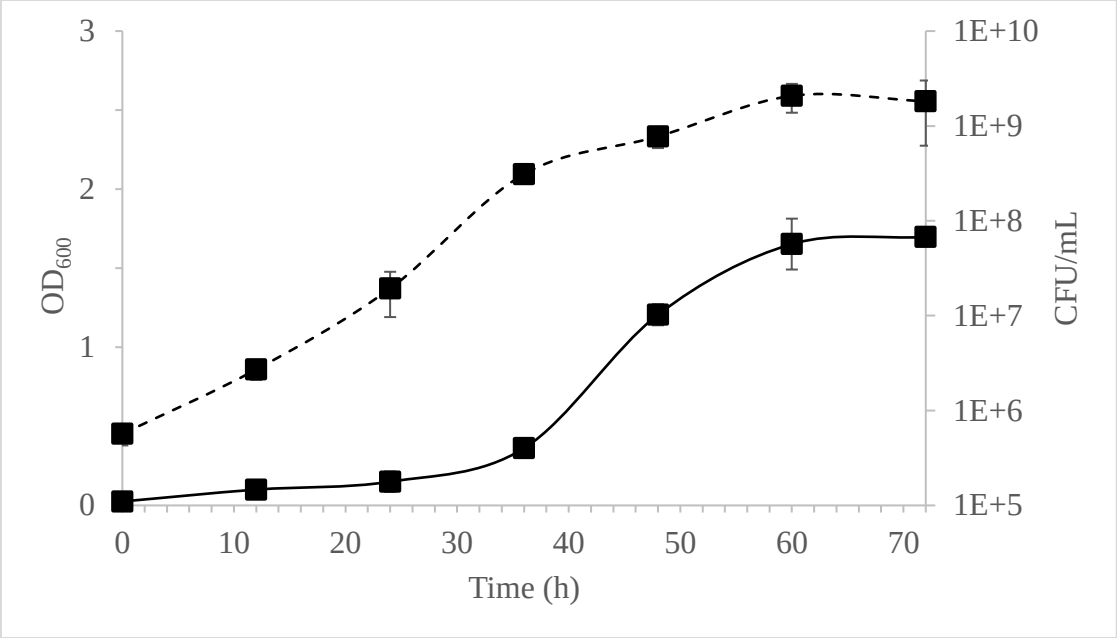

Figure 1. Growth of *Paucilactobacillus wasatchensis* WDC04 in MRS+Ribose broth (pH 6.5) at 23°C , as measured using plate counting (CFU/mL) (dashed line) and optical density (OD_{600}) (solid line), error bars=SE, n=3.

As seen in Figure 1, the apparent lag phase observed when growth is measured using OD occurs because prior to cell numbers reaching 10^8 CFU/mL, as determined by plate count, only a negligible increase in OD_{600} occurs. From this growth curve, an OD_{600} of 1.3 was determined to be equivalent to *Plb. wasatchensis* WDC04 culture being at a concentration of 10^9 CFU/mL.

Protective Cultures. Similar differences in the start of exponential growth as shown by measuring OD_{600} compared to counting bacterial numbers using plate counting was observed with the three protective cultures (Figures 2, 3, and 4). Although the non-observance of a lag phase based on bacterial numbers may have been because no plate count was performed between the time of inoculation and 6 h after inoculation while OD_{600} was measured every 3 h. Also, the protective cultures were inoculated at a level of 10^7 to 10^8 CFU/mL.

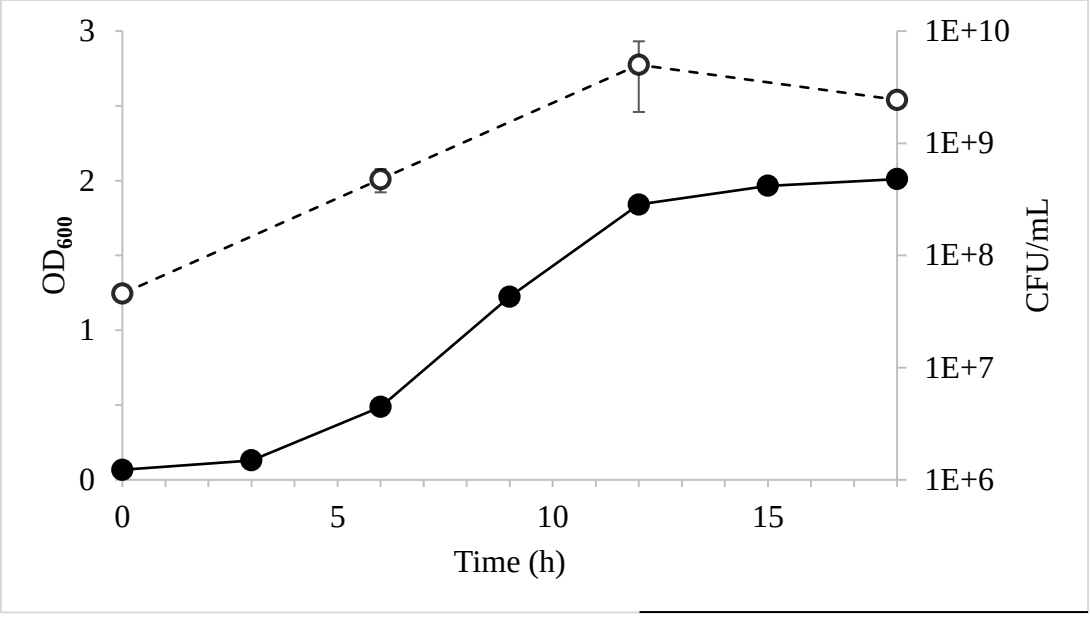

Figure 2. Growth of *Pediococcus acidilactici* 23F in MRS+Galactose broth pH 6.5 at 23°C , as measured using plate counting (CFU/mL) (open circle, dashed line) and optical density (OD_{600}) (closed circle, solid line), error bars=SE, n=3.

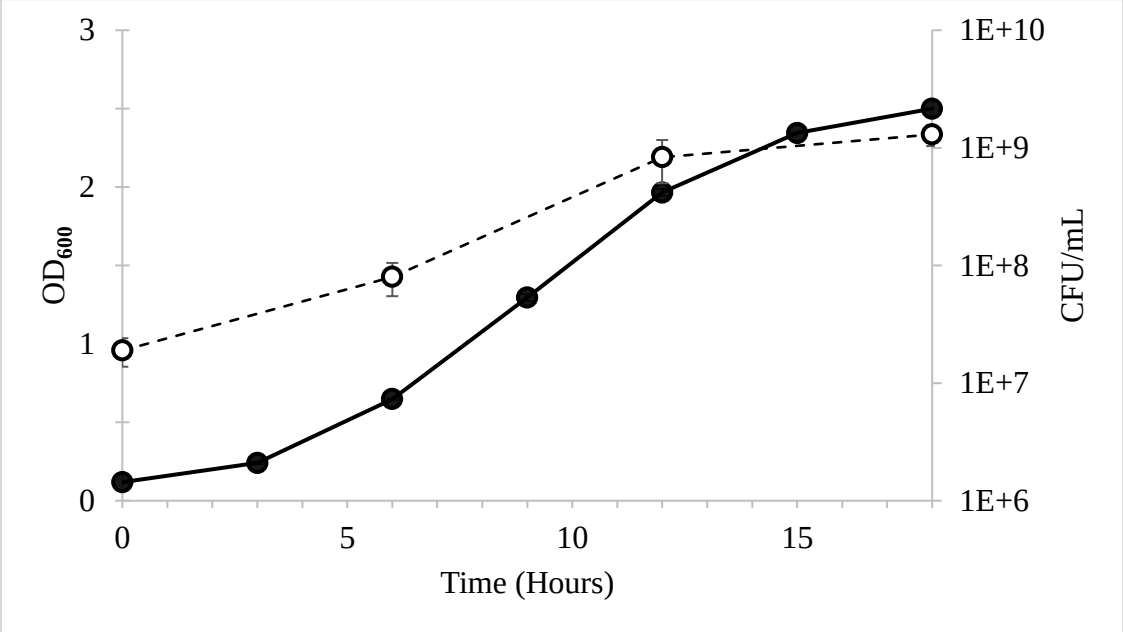

Figure 3. Growth of *Lactocaseibacillus casei* UW4 in MRS+Galactose broth pH 6.5 at 23°C , as measured using plate counting (CFU/mL) (open circle, dashed line) and optical density (OD_{600}) (closed circle, solid line), error bars=SE, n=3.

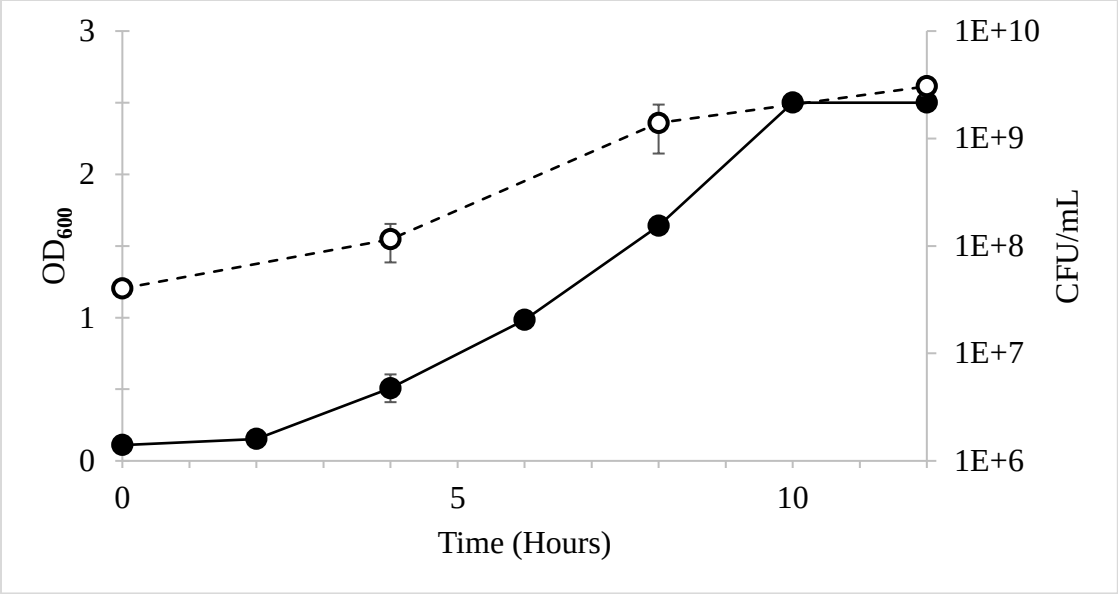

Figure 4. Growth of *Lactobacillus helveticus* 7995 in MRS+Galactose broth pH 6.5 at 23°C , as measured using plate counting (CFU/mL) (open circle, dashed line) and optical density (OD600) (closed circle, solid line), error bars=SE, n=3.

Based on OD_{600}, the lag phase for *Pc. acidilactici* 23F (Figure 2) and *Lcb. casei* UW4 (Figure 3) was 3 h while *Lb. helveticus* 7995 had a lag phase of only 2 h (Figure 4). Exponential growth as observed using OD_{600} varied for the 3 protective cultures and based on bacterial numbers from the plate counts, all three cultures reached 10^9 CFU/mL by 12 h of incubation. Plate count data showed that cultures *Pc. acidilactici* 23F (Figure 2) and *Lcb. casei* UW4 (Figure 3) had a much shorter lag phase and an exponential phase of 12 h; *Lb. helveticus* 7995 was observed to have a lag phase of 4 h and a log phase of the same amount of time (Figure 4).

From the growth curves (Figures 2, 3 and 4), it was observed that the OD_{600} value that corresponded to 10^9 CFU/mL differed slightly for the individual cultures and were 0.9, 2.19 and 1.35 respectively. However, these occurred within a time range of 8 to 12 h and so cultures were prepared for inoculation to be utilized in the next stage of the experiment after 10 h.

Model System for Testing Gas Production by Plb. wasatchensis

CR-MRS at pH 6.5. Gas production by *Plb. wasatchensis* WDC04 in the CR-MRS broth with different levels of added ribose and galactose was dependent on both the ratio of ribose:galactose and the inoculum level (Table 3A). More frequent gas production occurred at higher inoculum levels with gas bubbles observed in the Durham tubes between 6 and 16 d of incubation at 23ºC (Table 3B). That gas bubbles were not consistently produced in some treatment conditions is reflective of the sporadic observation of gas production in commercially manufactured cheese.

At low ribose levels (0.1% ribose:0.9% galactose), no gas production was observed and there was only minimal growth. This result concurs a previous observation of minimal *Plb. wasatchensis* WDC04 growth occurring when galactose is the only sugar (Ortakci et al., 2015). With a ribose:galactose ratio of 20:80 (0.2% ribose, 0.8% galactose), gas production depended on the *Plb. wasatchensis* WDC04 inoculum level, with no gas observed at the lowest inoculum levels (10^1 and 10^2 CFU/mL) but some gas production at the next higher inoculum levels. When this ratio of sugar was inoculated with 10^5 CFU/mL, gas was observed in all 3 replicates, but with higher inoculum levels, the frequency of gas production decreased. The most consistent gas production occurred in the ribose:galactose ratios of 40:60 (0.4% ribose:0.6% galactose), with a 10^6 CFU/mL inoculum, 30:70 (0.3% ribose:0.8% galactose) with inoculations of 10^5 or 10^6 CFU/mL, and with a 20:80 (0.2% ribose, 0.8% galactose) at a 10^5 CFU/mL inoculation. Ribose was required at a threshold level of 0.3% with 0.7% galactose for gas formation at the 10^2 CFU/mL to 10^4 CFU/mL inoculum levels. At higher inoculum levels (10^5-10^7 CFU/mL) only 0.2% ribose with 0.8% galactose was sufficient.

Table 3. **A.** Number of tubes gas production was observed in using triplicate tubes of CR-MRS[1] broth containing a total of 1% ribose (RIB) and galactose (GAL) at different ratios and with different inoculum levels of *Paucilactobacillus wasatchensis* WDC04, white = 0, light blue = 1, medium blue = 2, dark blue = 3 times. **B.** The time (d) at which gas was first observed in the Durham tubes (n=3).

A.

Sugar Ratio		Number of Times Gas Production Observed						
RIB	GAL	WDC04 Inoculum (CFU/mL)						
		10^1	10^2	10^3	10^4	10^5	10^6	10^7
50	**50**	0	1	2	0	2	2	2
40	**60**	2	2	2	2	2	3	2
30	**70**	0	2	2	2	3	3	2
20	**80**	0	0	1	1	3	2	2
10	**90**	0	0	0	0	0	0	0

B.

Sugar Ratio		Time When Gas Bubble Observed (d, d, d)						
		WDC04 Inoculum (CFU/mL)						
RIB	GAL	10^1	10^2	10^3	10^4	10^5	10^6	10^7
50	**50**	-[2], -, -	-, 10, -	-, 10, 10	-, -, -	11, 10, -	8, 10, -	8, 11, -
40	**60**	10, -, 11	-, 11, 10	-, 11, 12	-, 10, 15	-, 10, 12	10, 11, 8	10, -, 8
30	**70**	-, -, -	11, -, 11	-, 11, 11	-, 10, 10	11, 11, 8	15, 10, 11	10, -, 8
20	**80**	-, -, -	-, -, -	-, 10, -	-, -, 12	11, 8, 15	10, -, 12	10, 10, -
10	**90**	-, -, -	-, -, -	-, -, -	-, -, -	-, -, -	-, -, -	-, -, -

[1]Carbohydrate-restricted MRS broth with ribose and galactose the only sugars as described above
[2]No gas formation observed within 20 d. for that replicate

Gas was seen in Durham tubes in all three replicates for the 10^6 CFU/mL inoculum in 0.4% ribose:0.6% galactose tubes, for 10^5 and 10^6 CFU/mL inoculated into 0.3% ribose:0.7% galactose tubes, and for 10^5 CFU/mL inoculated into 0.2% ribose:0.8% galactose tubes. Inconsistent gas production was observed at the other combinations of inoculum level and sugar ratio (Table 3B).

Growth curves for the four combinations of *Plb. wasatchensis* WDC04 inoculum level and ribose:galactose ratio that consistently resulted in gas production are shown in Figure 5. Slightly faster growth occurred when more ribose was present (Table 4). The slowest growth rate (based upon μ_{max}) occurred with 0.2% ribose:0.8% galactose inoculated at 10^5 CFU/mL. The next slowest was the 0.3% ribose:0.7% galactose at both inoculum levels of 10^5 and 10^6 CFU/mL. The fastest growth rate was found with 0.4% ribose:0.6% galactose with an inoculum of 10^6 CFU/mL (Table 4). This showed a slightly faster growth occurring based upon available ribose and that the co-utilization of galactose with ribose as proposed by Ortakci et al. (2015a) was not exactly 1:1.

Only when CR-MRS broth contained 0.3% ribose:0.7% galactose, and was inoculated with 10^5 CFU/mL of *Plb. wasatchensis* WDC04, did gas production occur in all 5 of the additional replicates (Table 5). Under these conditions, the first observable gas occurred between 6 and 7 d of incubation. All other combinations of sugar ratios and inoculations under consideration for use as the model gas production system resulted in less consistent gas production and a broader time frame for the initial observation of gas. Maximum OD_{600} (**OD_{max}**) reached was similar for all four combinations (Table 6).

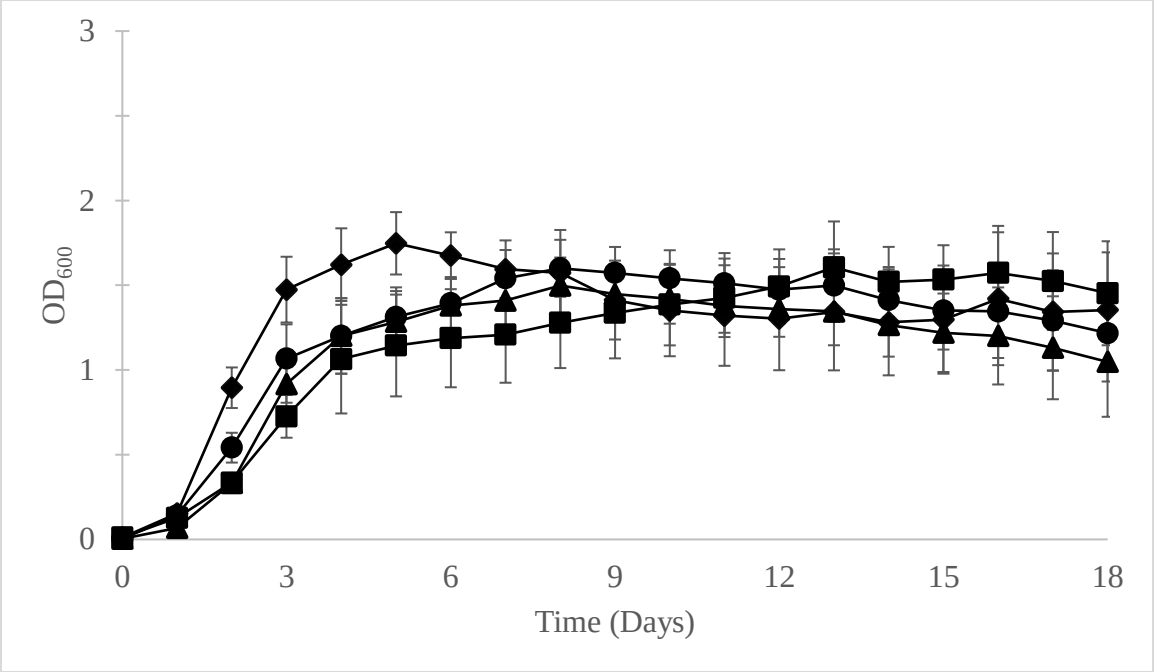

Figure 5. Growth at 23°C of *Paucilactobacillus wasatchensis* WDC04 inoculated at varying concentrations (CFU/mL) into carbohydrate-restricted MRS broth containing 1% sugar consisting of varying ratios of ribose:galactose: (■) 20:80 ratio with 10^5 CFU/mL, (▲) 30:70 ratio with 10^5 CFU/mL, (●) 30:70 ratio with 10^6 CFU/mL, and (♦) 40:60 ratio with 10^6 CFU/mL based on optical density (OD_{600}), error bars = SE, n = 5.

Table 4. Mean maximum specific growth rate (μmax) at 23°C of *Paucilactobacillus wasatchensis* WDC04 inoculated into carbohydrate-restricted MRS broth containing 1% sugar consisting of varying ratios of ribose:galactose observed to produce CO_2 as shown in Table 3, n=5.

Growth Condition	**μ_{max} ± SE**
Ribose:Galactose	OD_{600}/h
20:80 with 10^5 CFU/mL inoculum	0.016 ± 0.005
30:70 with 10^5 CFU/mL inoculum	0.024 ± 0.004
30:70 with 10^6 CFU/mL inoculum	0.022 ± 0.005
40:60 with 10^6 CFU/mL inoculum	0.031 ± 0.005

Table 5. Time (d) at 23°C until a gas bubble was initially observed in the Durham tube when *Paucilactobacillus wasatchensis* WDC04 was inoculated into carbohydrate-restricted MRS broth containing 1% sugar consisting of selected ratios of ribose:galactose from results found in Table 3 for each of 5 additional replicates.

	Growth Condition			
	Ribose:Galactose (Inoculum level (CFU/mL))			
Replicate	20:80 (10^5)	30:70 (10^5)	30:70 (10^6)	40:60 10^6
1	-[1]	7	9	6
2	16	6	6	8
3	6	6	6	6
4	7	6	7	9
5	-	7	-	-

[1]No gas formation observed over 21 d of incubation

Table 6. Mean (±SE) maximum optical density (OD_{max}) during incubation at 23°C of *Paucilactobacillus wasatchensis* WDC04 inoculated into carbohydrate-restricted MRS broth containing 1% sugar consisting of varying ratios of ribose:galactose and selected inoculum concentrations, n=5.

Growth Condition	
Ribose:Galactose (Inoculum level (CFU/mL))	OD_{max}
20:80 (10^5)	1.57 ± 0.28
30:70 (10^5)	1.50 ± 0.17
30:70 (10^6)	1.60 ± 0.17
40:60 (10^6)	1.75 ± 0.18

CR-MRS at pH 5.2 plus 3% NaCl. In order to more closely mimic a Cheddar cheese-like environment, experimental trials were run using the same ribose:galactose ratios added to CR-MRS that had been adjusted to pH 5.2 and with 3% NaCl added (Figure 6). It was observed that after the OD_{max} was reached in each growth condition, interestingly, there was a drop in OD. This drop was steepest and largest when ribose was the only sugar and decreased as the galactose concentration in the ratio was increased. When OD_{600} was observed over a long period of time (60 d) there was a slow increase in OD_{600} with the higher levels of ribose and either no increase (or even a decrease) as the proportion of galactose increased.

Growth rates as determined by μ_{max} were similar when at least 30% of the added sugar was ribose (Table 7). Growth rate was about one third slower with the 20:80 media and about two thirds slower when only 10% of the added sugar was ribose. The μ_{maxc} was similar for ribose:galactose ratios 100:0, 50:50. 40:60, and 30:70 at initial inoculum concentrations of 10^7, 10^5, 10^3, and 10^1 CFU/mL; as well as, 20:80 at 10^5 CFU/mL (Table 7). Growth slowed down for carbohydrate ratios of 20:80 when using inoculum concentrations of 10^7, 10^3, or 10^1 CFU/mL and for the sugar ratio of 10:90 at inoculum concentrations of 10^7, 10^5, 10^3, or 10^1 CFU/mL. The OD_{max} follow a trend of becoming lower than the previous initial inoculum level within the same ribose:galactose ratio, with two exceptions, a sugar ratio of 100:0 at an inoculum concentration of 10^3 CFU/mL and the sugar ratio of 40:60 at an inoculum concentration of 10^1 CFU/mL (Table 8). As the ribose decreased and the galactose increased, there was also a trend of lower OD_{max} values apart from the combinations of 50:50 and 10^5 CFU/mL, and 40:60 and 10^1 CFU/mL (Table 8). These observed decreases in μmax, as well as OD_{max}, may be what

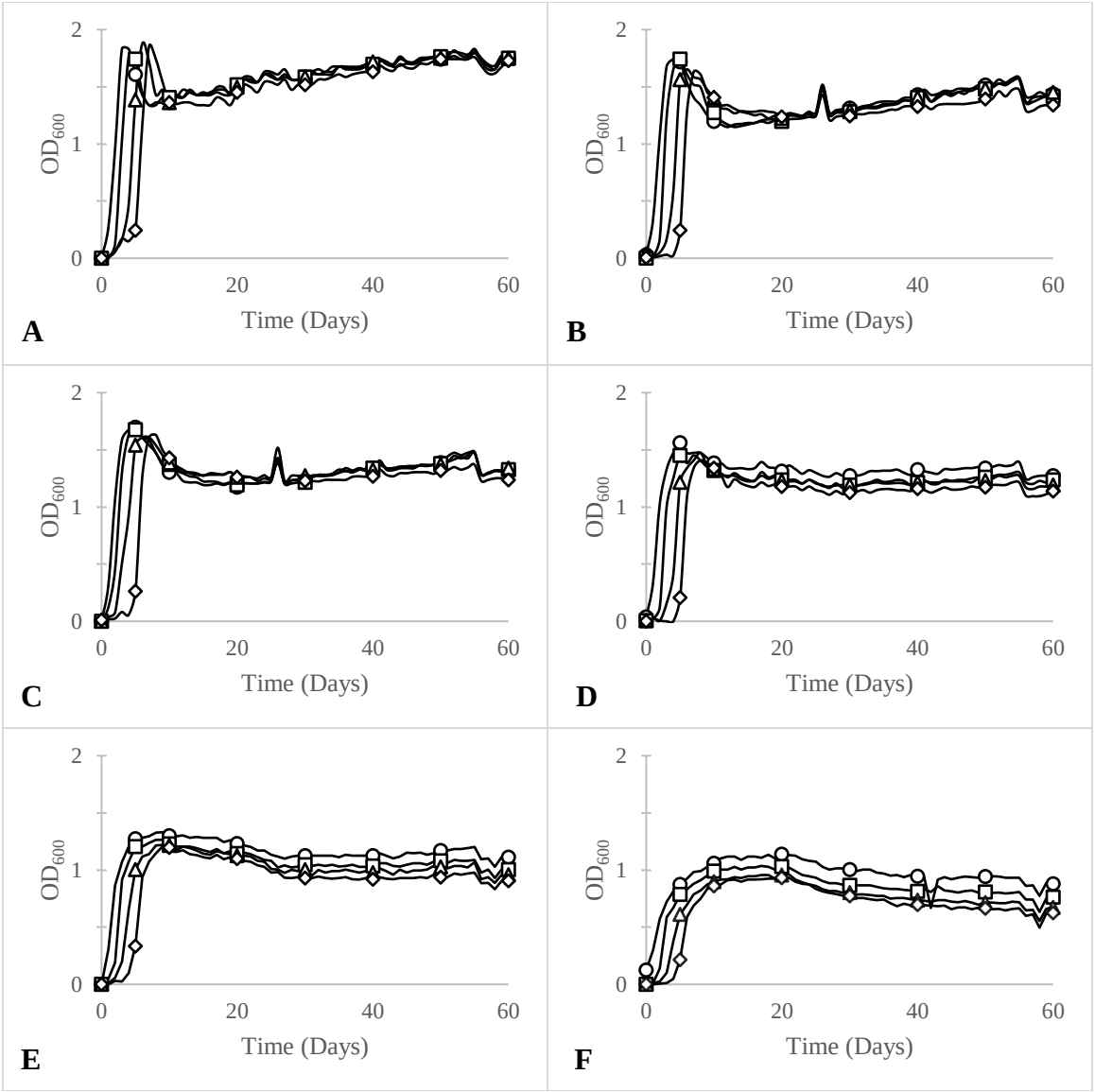

Figure 6. Growth at 23°C of *Paucilactobacillus wasatchensis* WDC04 in carbohydrate-restricted MRS (pH 5.2) with 3% NaCl added at ribose:galactose ratios (1% total sugar) of 100:0 (A), 50:50 (B), 40:60 (C), 30:70 (D), 20:80 (E), 10:90 (F). For each ratio of sugars, initial inoculum was 10^1 ( ), 10^3 ( ), 10^5 ( ), and 10^7 ( ) CFU/mL.

Table 7. The maximum specific growth (μ_{max}) at 23°C of *Paucilactobacillus wasatchensis* WDC04 in carbohydrate-restricted MRS with various ribose:galactose ratios inoculated initially at concentrations of 10^7,10^5,10^3, and 10^1 CFU/mL, n =3.

	μ_{max}			
Ribose:Galactose[1]	Inoculum Level			
	10^7	10^5	10^3	10^1
100:0	0.036	0.038	0.038	0.038
50:50	0.031	0.044	0.041	0.043
40:60	0.032	0.035	0.026	0.042
30:70	0.029	0.036	0.034	0.036
20:80	0.023	0.030	0.019	0.025
10:90	0.011	0.019	0.012	0.016

[1] 1% total sugar concentration

Table 8. The maximum optical density (OD_{max}) of *Paucilactobacillus wasatchensis* WDC04 in carbohydrate-restricted MRS at 23°C with various ribose:galactose ratios inoculated initially at levels 10^7,10^5,10^3, and 10^1 CFU/mL. ± Error=SE, n=3.

	OD_{max}			
Ribose:Galactose[1]	Inoculum Level (CFU/mL)			
	10^7	10^5	10^3	10^1
100:0	1.83 ± 0.02	1.76 ± 0.02	1.88 ± 0.00	1.85 ± 0.02
50:50	1.74 ± 0.00	1.74 ± 0.00	1.65 ± 0.02	1.63 ± 0.02
40:60	1.70 ± 0.02	1.68 ± 0.00	1.61 ± 0.00	1.63 ± 0.02
30:70	1.56 ± 0.02	1.45 ± 0.02	1.47 ± 0.00	1.41 ± 0.04
20:80	1.33 ± 0.02	1.26 ± 0.01	1.21 ± 0.01	1.21 ± 0.00
10:90	1.14 ± 0.02	1.04 ± 0.00	0.96 ± 0.01	0.93 ± 0.01

[1] 1% total sugar concentration

caused *Plb. wasatchensis* WDC04 to show variable gas production or none at all in previous experiments. However, no gas appeared in any of the Durham tubes in these experiments after 60 d.

Protective cultures inhibition of Plb. wasatchensis WDC04

Optical Density. Optical density measurements of *Plb. wasatchensis* WDC04 followed the pattern of growth from the model system experiments in GPB with starting inoculum level 10^5 CFU/mL (Figure 7). Gas production is indicated with an X. This reinforces the repeatability of the model system as a control. All three protective cultures alone, and when grown with *Plb. wasatchensis* WDC04 (regardless of initial protective inoculum level) reached OD_{max} of the spectrophotometer (2.5) by 4 d and maintained this level until the end of the 12-d experiment (Figures 8, 9 and10). Due to this observation, growth of all three protective cultures appears to reach OD_{max} at 4 d. This may not be accurate as OD readings may be higher, revealing the limitations of total growth measurements using this technique. Optical density was not a sufficient measure of growth of protective cultures with *Plb. wasatchensis* WDC04 since growth cannot be separately tracked for each culture. For these reasons, plate counts were used in combination with optical density to differentiate cell growth between *Plb. wasatchensis* WDC04 when grown together.

Plate Counts (CFU/mL). Regardless of inoculum level with *Plb. wasatchensis* WDC04 or in pure culture, the protective cultures all reached maximum growth of ~10^9 CFU/mL by 4 d (Figures 11 to 14). *Pediococcus acidilactici* 23F had decreased to a level of ~10^8 CFU/mL from 4 to 8 d, then maintained this cell population until the end of the experiment (Figure 12). *Lacticaseibacillus casei* UW4 maintained a cell population of

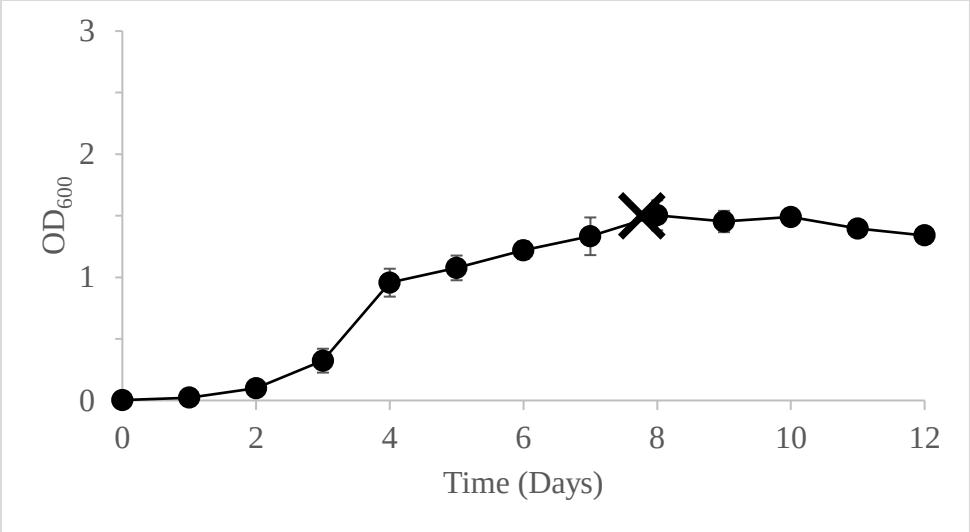

Figure 7. Growth of *Paucilactobacillus wasatchensis* WDC04 at initial inoculum concentration of 10^5 CFU/mL (●) in carbohydrate-restricted MRS at 23°C was measured at OD_{600} with a spectrophotometer. Initial gas production is indicated with an X. Error bars=SE, n=3.

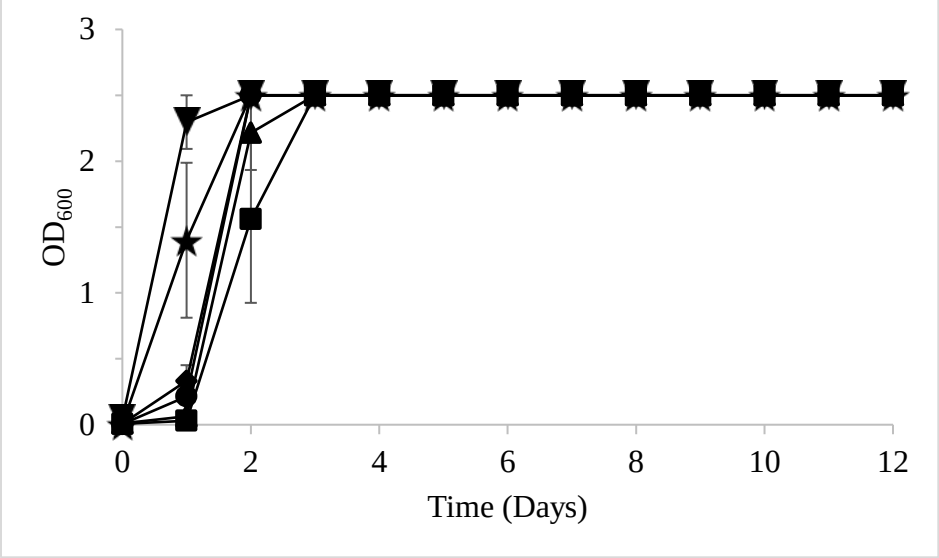

Figure 8. Growth at 23°C of Pediococcus acidilactici 23F was observed alone at an initial concentration of 105 CFU/mL (●) or with Paucilactobacillus wasatchensis WDC04 (initially added at 105 CFU/mL) at initial concentrations of 103 (■), 104 (▲), 105 (♦), 106 (★), or 107 (▼) CFU/mL protective culture using OD600. Error=SE. Control n=2, treatment n=3.

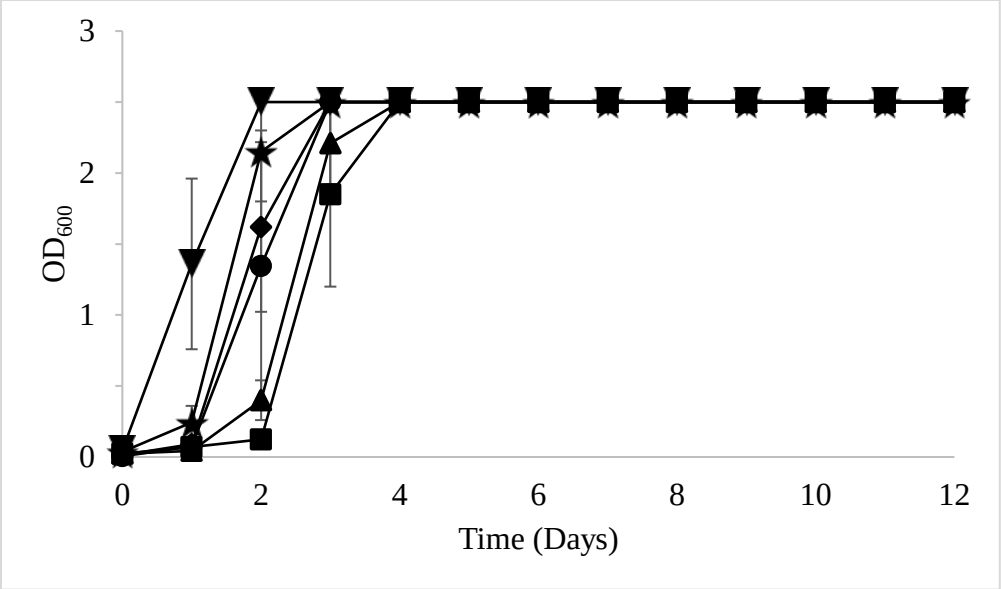

Figure 9. Growth at 23°C of *Lacticaseibacillus casei* UW4 was observed alone at an initial concentration of 10^5 CFU/mL (●) or with *Paucilactobacillus wasatchensis* WDC04 (initially added at 10^5 CFU/mL) at initial concentrations of 10^3 (■), 10^4 (▲), 10^5 (♦), 10^6 (★), or 10^7 (▼) CFU/mL protective culture using OD_{600}. Error=SE. Control n=2, treatment n=3.

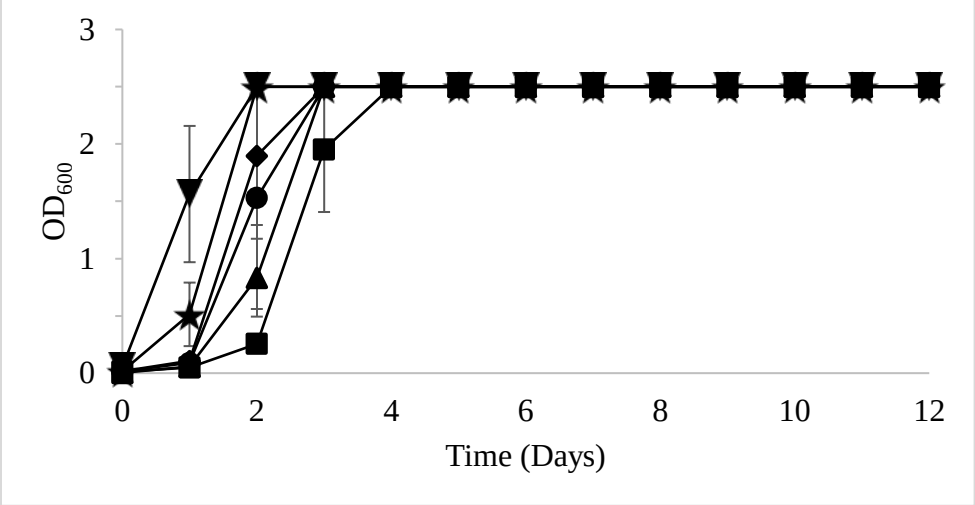

Figure 10. Growth at 23°C of *Lactobacillus helveticus* 7995 was observed alone at an initial concentration of 10^5 CFU/mL (●) or with *Paucilactobacillus wasatchensis* WDC04 (initially added at 10^5 CFU/mL) at initial concentrations of 10^3 (■), 10^4 (▲), 10^5 (♦), 10^6 (★), or 10^7 (▼) CFU/mL protective culture using OD_{600}. Error=SE. Control n=2, treatment n=3.

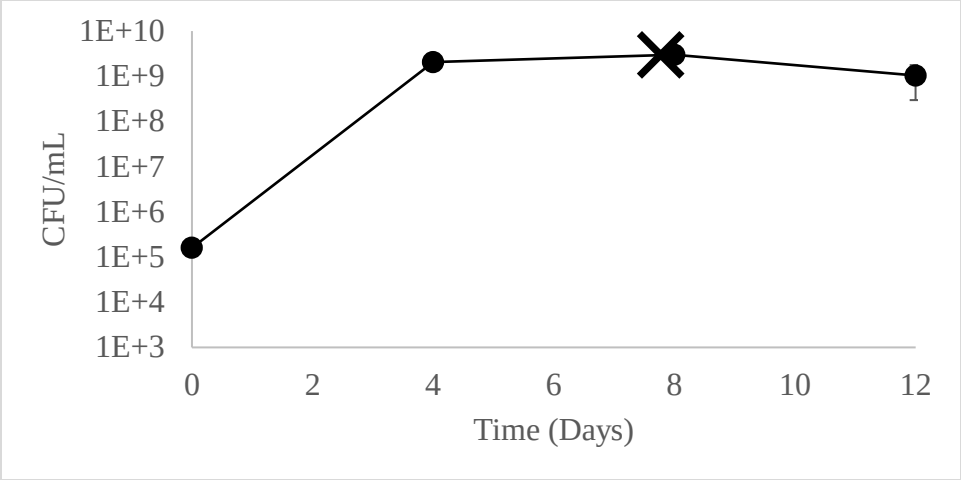

Figure 11. Growth of *Paucilactobacillus wasatchensis* WDC04 at an initial inoculum level 10^5 CFU/mL (●) was measured in CFU/mL using plate counts on MRS+R spread plates. Initial observations of gas production is indicated by an X. Error bars=SE, n=3.

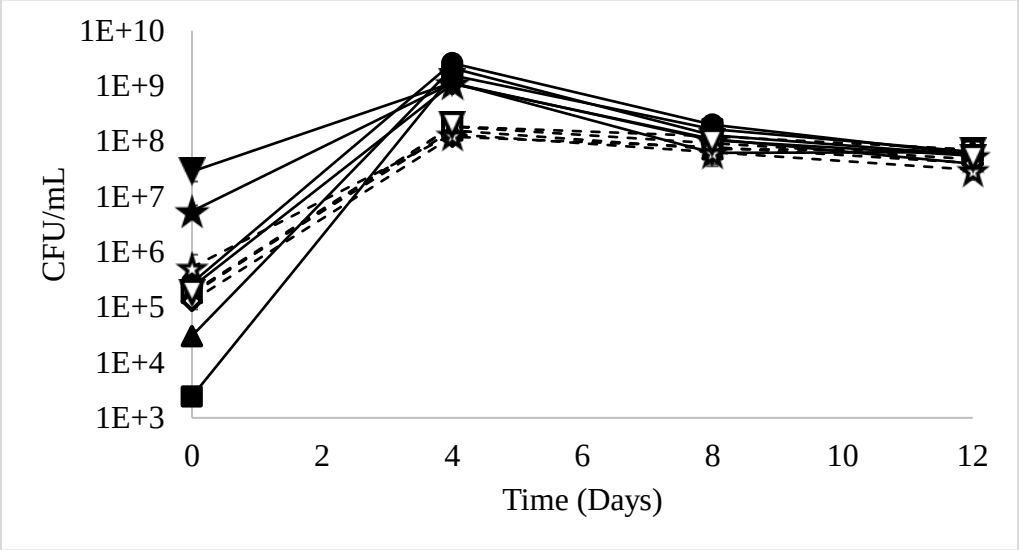

Figure 12. Growth of Pediococcus acidilactici 23F (solid line, closed symbol) was observed alone at an initial concentration of 105 CFU/mL (●) or with Paucilactobacillus wasatchensis WDC04 (initially added at 105 CFU/mL; dashed line, open symbol) at initial concentrations of 103 (■), 104 (▲), 105 (♦), 106 (★), or 107 (▼) CFU/mL. Protective culture was measured in CFU/mL on MRS+R spread plates. Error=SE. Control n=2, treatment n=3.

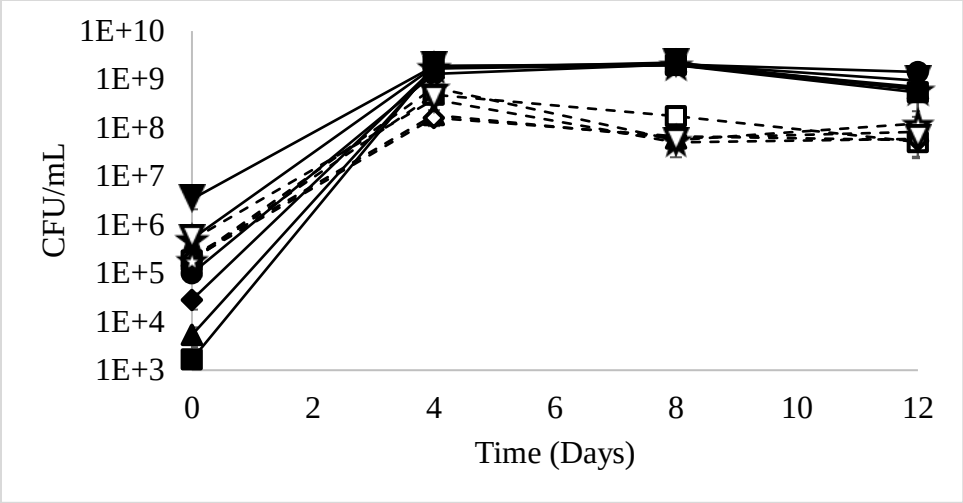

Figure 13. Growth of Lacticaseibacillus casei UW4 (solid line, closed symbol) was observed alone at an initial concentration of 105 CFU/mL (●) or with Paucilactobacillus wasatchensis WDC04 (initially added at 105 CFU/mL; dashed line, open symbol) at initial concentrations of 103 (■), 104 (▲), 105 (♦), 106 (★), or 107 (▼) CFU/mL. Protective culture was measured in CFU/mL on MRS+R spread plates. Error=SE. Control n=2, treatment n=3.

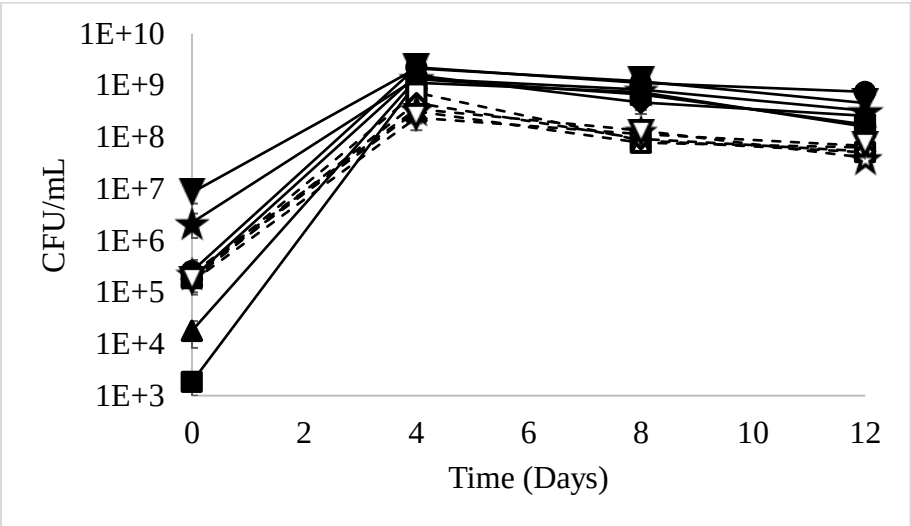

Figure 14. Growth of Lactobacillus helveticus 7995 (solid line, closed symbol) was observed alone at an initial concentration of 105 CFU/mL (●) or with Paucilactobacillus wasatchensis WDC04 (initially added at 105 CFU/mL; dashed line, open symbol) at initial levels of 103 (■), 104 (▲), 105 (♦), 106 (★), or 107 (▼) CFU/mL. Protective culture was measured in CFU/mL on MRS+R spread plates. Error=SE. Control n=2, treatment n=3.

~10^9 CFU/mL for the entirety of the experiment (Figures 13). Interestingly, in *Lb. helveticus* 7995, the control with initial inoculum maintained a population of ~10^9 the entire experiment (Figure 14). The test tubes containing *Plb. wasatchensis* WDC04 however, decreased steadily to the cell population of ~10^8 by 12 d (Figure 11).

Observations for the control *Plb. wasatchensis* WDC04 phase of the experiment with a cell population of ~10^9 CFU/mL being reached by d 4 and maintained for the rest of the experiment (Figure 11). Gas production was observed in the Durham tube in test tubes containing only *Plb. wasatchensis* WDC04. No gas was observed in test tubes containing both *Plb. wasatchensis* WDC04 and a protective culture during the entire reach ~10^8 CFU/mL regardless of the initial protective culture inoculum concentration experimental time period. When plates were made containing both protective culture and *Plb. wasatchensis* WDC04, the counts of *Plb. wasatchensis* WDC04 was only able toreach ~10^8 cfu/mL regardless of the initial protective culture inoculum concentration (Figures 12 to 14). This reduction in maximum cell number may be a factor in the inability of *Plb. wasatchensis* WDC04 to produce gas in test tubes also containing a protective culture. Reduction in *Plb. wasatchensis* WDC04 cell number when in test tubes with protective cultures is likely due to competition for nutrients or the protective cultures' ability to inhibit *Plb. wasatchensis* WDC04 in other ways.

Statistical analysis was done to determine whether the growth of *Plb. wasatchensis* WDC04 grown with a protective culture was significantly different from *Plb. wasatchensis* WDC04 in pure culture (Appendix A, Table 9). *Paucilactobacillus wasatchensis* WDC04 alone was significantly different from its growth with a protective culture on d 4, 8 and 12; but not initially. On d 4 and 12, there were significant

differences among the level of *Plb. wasatchensis* WDC04 in media with all three protective cultures. In addition, a significant difference was also observed between *Plb. wasatchensis* WDC04 grown with *Pc. acidilactici* 23F or with *Lb. helveticus* 7995 on d 4. The final day of the experiment showed significant differences between *Plb. wasatchensis* WDC04 grown with *Pc. acidilactici* 23F and *Lcb. casei* UW4.

Galactose

Megazyme Standard Curve. From each estimated percentage of galactose added to CR-MRS broth, the percentage of galactose was calculated according to the ΔAD-galactose = [(A2-A1)galactose sample - (A2-A1)galactose blank] * dilution factor formula suggested in the Megazyme kit. The R^2 value (0.9991) obtained from the standard curve shows that the actual and calculated percentage of galactose values are very closely related (Figure 15). Thus, the formula mentioned above was used for all further galactose percentage determinations from the absorbance values detected from use of the Megazyme galactose kit.

Galactose. Initial galactose concentrations were observed at levels 0.04 to 0.1% less than the intended 0.7% carbohydrate in prepared CR-MRS broth. This decrease was due to the requirement of putting the carbohydrates in the test tubes prior to autoclaving. Balancing the Durham tube on a capillary tube and inserting that into a test tube must be done prior to autoclaving. This limited the addition of substrates post autoclave because the capillary tubes were quite fragile, so vortexing the test tubes to mix in added substrates was not an option. Vortexing can also unintentionally introduce gas into the Durham tube prior to inoculation and incubation

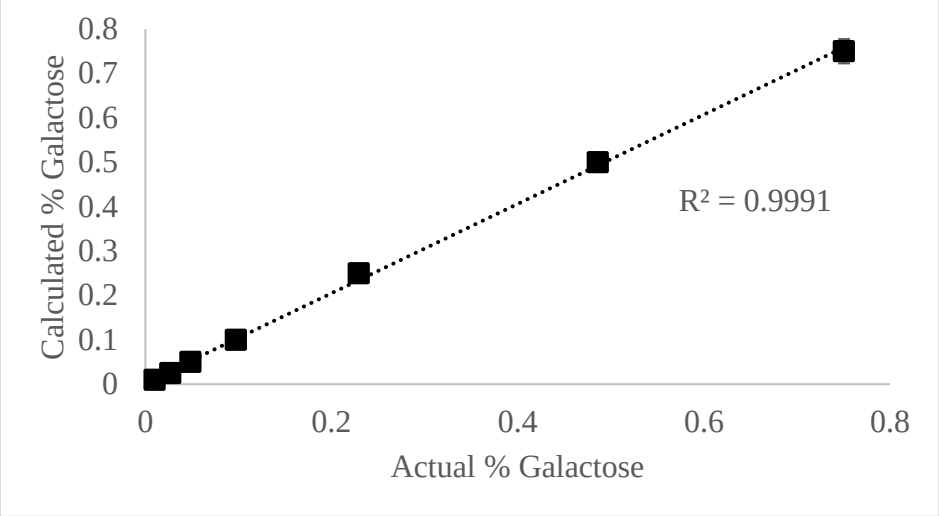

Figure 15. A standard curve was created CR-MRS broth with concentrations of 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 0.75% wt/vol galactose in duplicate. The y-axis indicates the percentage of galactose added to the CR-MRS. The x-axis (Actual % Galactose) was calculated using the formula; ΔAD-galactose = [(A2-A1)galactose sample - (A2-A1)galactose blank] * dilution factor. A trendline was used to determine an R^2 value. Standard error was determined for the calculated galactose values.

Previous work concluded that *Plb. wasatchensis* WDC04 co-utilizes galactose and ribose (Ortakci, et al., 2015a). This research was able to track the galactose being utilized as *Plb. wasatchensis* WDC04 grew and gas was produced. Gas was produced in 7.7 ds on average in test tubes containing only *Plb. wasatchensis* WDC04. The galactose measurement at 8 d was observed to be only 0.15%. Galactose was observed to be steadily utilized over the course of the 12 d by *Plb. wasatchensis* WDC04, rather than completely depleted by 4 d as seen in the protective culture test tubes. By 12 d, galactose in the test tube containing only *Plb. wasatchensis* WDC04 was exhausted.

The protective cultures at an initial concentration of 10^7 CFU/mL with *Plb. wasatchensis* WDC04 added at initial concentration of 10^5 CFU/mL reduced the galactose concentration to 0.32 to 0.47% by the end of d 1. Each protective culture at all initial concentrations of inoculum (10^3-10^7 CFU/mL), with or without added *Plb.*

wasatchensis WDC04 had no detectable galactose by d 4 (Figures 16 to 19). The depletion of galactose in the mixed culture occurred either faster or at the same pace as galactose depletion in test tubes containing only the protective culture. Therefore, galactose utilization by the protective cultures was not inhibited by the addition of *Plb. wasatchensis* WDC04.

Gal+/Lac+ Protective Culture

Protective culture *Pediococcus pentosaceus* FBB61 was determined to utilize lactose on the API 50 CH panel but not in the initial test using Bromocresol Purple Agar with lactose added. So, although this protective culture wasn't originally part of the *Plb. wasatchensis* WDC04 inhibition experiments, it was also subjected to the same experimentation alongside the Gal+/Lac- protective cultures. A growth curve was done using the same format as the *Lb. helveticus* 7995 curve. The OD_{600} at which 10^9 CFU/mL was attained was 2.0 (Appendix B, Figure 20).

The inhibition of *Plb. wasatchensis* WDC04 with *Pc. pentosaceus* FBB61 added at concentrations 2 logs higher or lower than 10^5 CFU/mL showed a pattern similar to the other protective cultures in regard to OD (reaching maximum). However, the $OD_{\mathbf{max}}$ was observed for all concentrations of *Pc. pentosaceus* FBB61 with or without *Plb. wasatchensis* WDC04 by 2 d rather than 4 d (Appendix B, Figure 21). Plate count data showed that *Pc. pentosaceus* FBB61, regardless of the initial inoculum concentration or the presence of *Plb. wasatchensis* WDC04, reached an OD with an average concentration of ~10^9 CFU/mL by 4 d and remained at this concentration throughout the remainder of the experiment. *Paucilactobacillus wasatchensis* WDC04 grew to a concentration of ~10^8

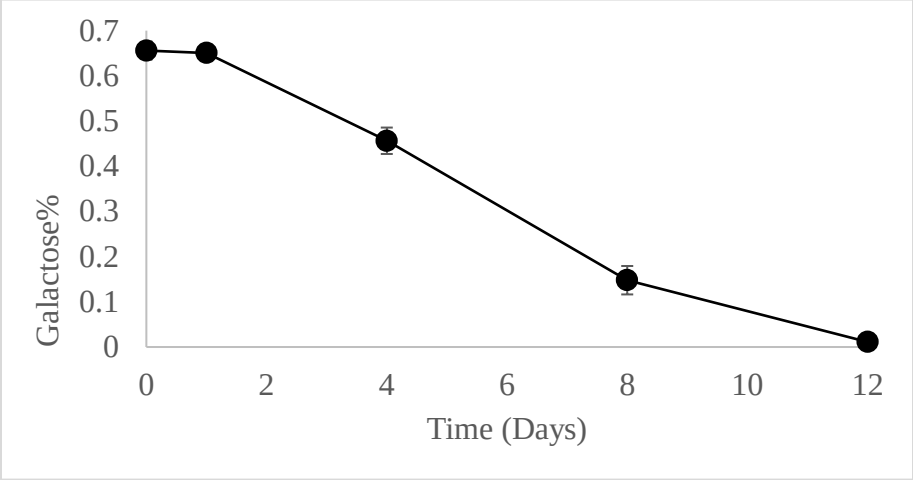

Figure 16. Galactose utilization by *Paucilactobacillus wasatchensis* WDC04 at an initial inoculum concentration of 10^5 CFU/mL (●) was measured by percent galactose using an enzymatic method at 0, 1, 4, 8, and 12 d. Error bars=SE, n=3.

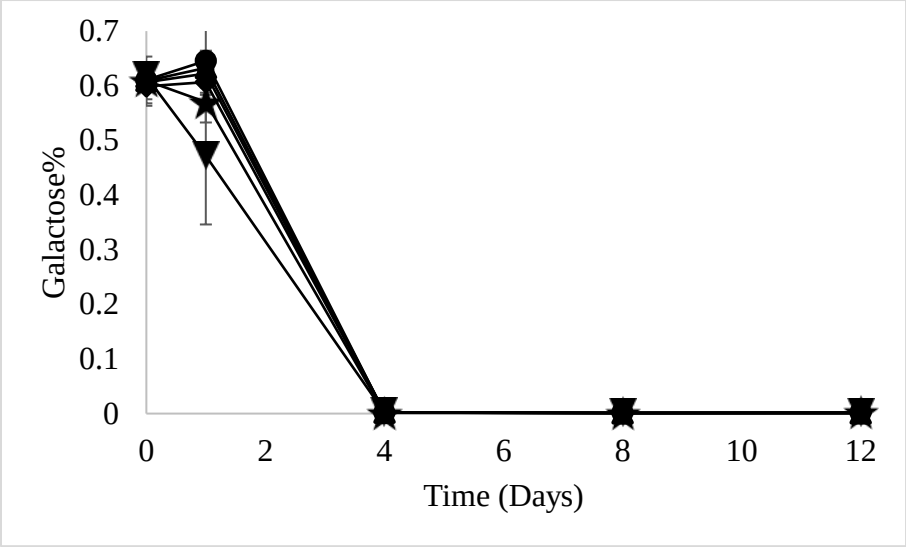

Figure 17. Galactose utilization by *Pediococcus acidilactici* 23F was observed alone at an initial concentration of 10^5 CFU/mL (●) or with *Paucilactobacillus wasatchensis* WDC04 (initially added at 10^5 CFU/mL) at initial concentrations of 10^3 (■), 10^4 (▲), 10^5 (♦), 10^6 (★), or 10^7 (▼) CFU/mL was measured by percent galactose using an enzymatic method at 0, 1, 4, 8, and 12 d. Error=SE. Control n=2, treatment n=3.

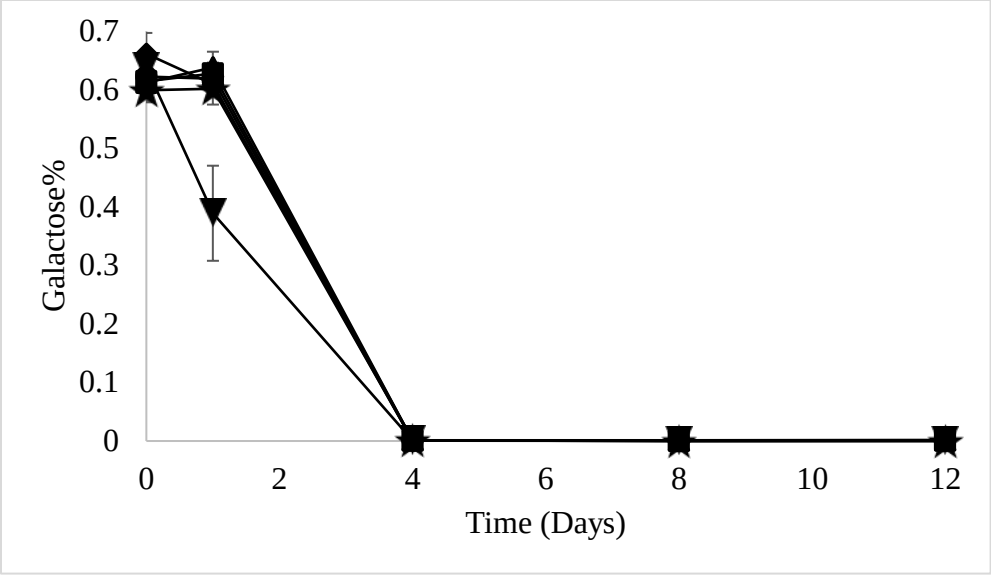

Figure 18. Galactose utilization by Lacticaseibacillus casei UW4 was observed alone at an initial concentration of 105 CFU/mL (●) or with Paucilactobacillus wasatchensis WDC04 (initially added at 105 CFU/mL) at initial levels of 103 (■), 104 (▲), 105 (♦), 106 (★), or 107 (▼) CFU/mL was measured by percent galactose using an enzymatic method at 0, 1, 4, 8, and 12 d. Error=SE. Control n=2, treatment n=3.

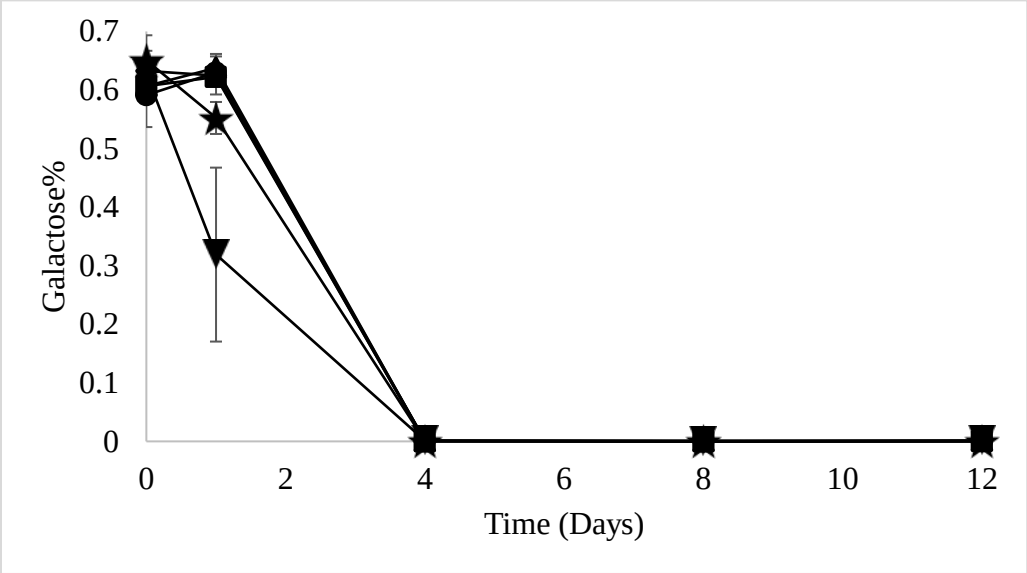

Figure 19. Galactose utilization by *Lactobacillus helveticus* 7995 was observed alone at an initial concentration of 10^5 CFU/mL (●) or with *Paucilactobacillus wasatchensis* WDC04 (initially added at 10^5 CFU/mL) at initial levels of 10^3 (■), 10^4 (▲), 10^5 (♦), 10^6 (★), or 10^7 (▼) CFU/mL was measured by percent galactose using an enzymatic method at 0, 1, 4, 8, and 12 d. Error=SE. Control n=2, treatment n=3.

CFU/mL by 4 d and also remained at that concentration (Appendix B, Figure 22). This growth pattern was similar to that of protective culture *Lcb. casei* UW4.

Galactose measurements obtained from the *Plb. wasatchensis* WDC04 inhibition experiments, like OD_{600}, showed a pattern similar to all the protective cultures. Regardless of the presence of *Plb. wasatchensis* WDC04 or the initial amount of culture added, galactose had been completely depleted by 4 d (Appendix B, Figure 23). There was also no gas formation in any test tubes containing Durham tubes balanced on a capillary tube.

CONCLUSION

By inoculating *Plb. wasatchensis* WDC04 into CR-MRS broth containing various ratios of ribose and galactose (1% total sugar wt/vol) it was possible to determine conditions under which *Plb. wasatchensis* WDC04 always produced observable gas in a Durham tube. Elevating the Durham tube by placing it upon a capillary tube increased the volume of broth from which gas production could be captured. The inoculum concentration of *Plb. wasatchensis* WDC04 (CFU/mL) paired with the ratio of ribose:galactose played an important role in determining whether gas formation occurred in test media. The pH and salt concentration in the test media also appeared to influence gas formation. At pH 6.5, the first set of experiments determined there were four growth conditions, ribose:galactose ratios and inoculum concentrations, in which *Plb. wasatchensis* WDC04 was able to produce gas every time. Five subsequent confirmatory experimental replicates further narrowed gas production conditions to an initial inoculum concentration of 10^5 CFU/mL and a ribose:galactose ratio of 30:70. This CR-MRS broth containing this sugar ration was then designated as the Gas Production Broth. Reproducible gas production occurred when 10^5 CFU/mL of *Plb. wasatchensis* WDC04 was inoculated into CR-MRS broth (pH 6.5) with 1% sugar added as a 30:70 mixture of ribose to galactose. This method was designated the gas production test (GPT).

The overall objective of this project was to determine if the addition of a protective LAB adjunct culture to metabolize galactose by could inhibit gas production by *Plb. wasatchensis* WDC04. This hypothesis was observed to be correct. Forty potential LAB cultures were tested for carbohydrate utilization with an emphasis on galactose and lactose fermentation. From these forty, three Gal+/Lac- cultures were

selected for further investigation; *Lacticaseibacillus casei* UW4, *Lactobacillus helveticus* 7995, and *Pediococcus acidilacti* 23F.

Growth and galactose utilization by the three protective cultures either in the presence or absence of *Plb. wasatchensis* WDC04 was very similar with galactose completely depleted by 4 d. Gas formation by *Plb. wasatchensis* WDC04 in the gas production test did not occur if a protective culture was present. Growth of *Plb. wasatchensis* WDC04 was 1.5 log less when grown in the presence of a protective culture than in pure culture. When grown without added protective cultures, galactose was steadily exhausted by *Plb. wasatchensis* WDC04 by 12 d and at 8 d gas was initially observed in the Durham tube while the galactose concentration had decreased to 0.15%.

The model system that was developed can be used for testing gas production by other OHF NSLAB. Adding a culture such as *Pc.s acidilactici* 23F, *Lcb. casei* UW4 or *Lb. helveticus* 7995 that are Lac^- but Gal^+ quickly depleted galactose and prevented its utilization by *Plb. wasatchensis* WDC04 to produce CO_2. Such cultures have potential to be used as protective adjunct cultures in cheese manufacture to prevent unwanted gas production, blowing and slits and cracks in the cheese.

REFERENCES

Bley, M. E., M. E. Johnson, and N. F. Olsen. 1985. Factors affecting nonenzymatic browning of process cheese. J. Dairy Sci. 68:555-561. https://doi.org/10.3168/jds.S0022-0302(85)80858-4.

Broadbent, J. R., K. Houck, M. E. Johnson and C. J. Oberg. 2003. Influence of adjunct use and cheese microenvironment on nonstarter bacteria in reduced-fat Cheddar-type cheese. J. Dairy Sci. 86:2773-2782. http://dx.doi.org/10.3168/jds.S0022-0302(03)73874-0.

Crowe, V. L., T. Coolbear, P. K. Gopal, F. G. Martley, L. L. McKay, and H. Riepe. 1995. The role of autolysis of lactic acid bacteria in the ripening of cheese. Int. Dairy J. 5: 855-875. http://dx.doi.org/10.1016/0958-6946(95)00036-4.

Culumber, M., D. J. McMahon, F. Ortakci, L. Montierth, B. Villalba, J., R. Broadbent, and C. J. Oberg. 2017. Geographical distribution and strain diversity of *Lactobacillus wasatchensis* isolated from cheese with unwanted gas formation. J. Dairy Sci. 100:8764-87-67. https://doi.org/10/10.3168/jds.2017-13375.

Fox, P. F., P. L. H. McSweeney, and C.M. Lynch. 1998. Significance of non-starter lactic acid bacteria in Cheddar cheese. Aust. J. of Dairy Technol. 53:83-89.

Jordan, K. N., and T. M. Cogan. 1993. Identification and growth of non-starter lactic acid bacteria in Irish Cheddar cheese. Irish J Agri. Food Res. 32:47-55. https://www.jstor.org/stable/25562201.

Lawrence, R. C., H. A. Heap, and J. Gilles. 1984. A controlled approach to cheese technology. J. Dairy Sci. 67: 1632-1645. http://dx.doi.org/10.3168/jds.S0022-0302(84)81486-1.

Lowrie, R.J. and R.C. Lawrence. 1972. Cheddar Cheese Flavor. IV. A new hypothesis to account for the development of bitterness. N. Z. J. Dairy Sci. Technol. 7:51-54.

Lowrie, R. J., R. C. Lawrence, and M. F. Peberdy. 1974. Cheddar cheese flavor. V. Influence of bacteriophage and cooking temperature on cheese under bacteriological conditions. N. Z. J. Dairy Sci. Technol. 9:116-121.

McMahon, D. J., C. J. Oberg, M. A. Drake, N. Farkye, L. V. Moyes, M. R. Arnold, B. Ganessan, J. Steele, and J. R. Broadbent. 2014. Effect of sodium, potassium, magnesium, and calcium salt cations on pH, proteolysis, organic acids, and microbial populations during

storage of full-fat Cheddar cheese. J. Dairy Sci. 97: 4780-4798. http://dx.doi.org/10.3168/jds.2014-8071.

Michel, V., and F. G. Martley. 2001. *Streptococcus thermophilus* in Cheddar cheese – production and fate of galactose. J. Dairy Res. 68:317-325.

Montville, T. J. and K. R. Matthews. 2005. Food microbiology: an introduction. ASM Press. Washington DC.

Oberg, C. J., L. V. Moyes, M. J. Domek, C. Brothersen, and D. J. McMahon. 2011. Survival of probiotic adjunct cultures in cheese and challenges in their enumeration using selective media. J. dairy Sci. 94:2220-2230. http://dx.doi:10:3168/jds.2010-3934.

Oberg, C. J., T. S. Oberg, M.C. Culumber, F. Ortakci, J. R. Broadbent, and D. J. McMahon. 2016. *Lactobacillus wasatchensis* sp. nov., a non-starter lactic acid bacteria isolated from aged Cheddar cheese. Int. J. of Systematic and Evolutionary Micro. 66:158-164. http://dx.doi.org/10.1099/ijsem.0.000689.

Orsi, C., and A.V. Zambrini. 2017. Starter and Ancillary Cultures. Advances in Dairy Products. 58–74. http://dx.doi.org/10.1002/9781118906460.ch1d.

Ortakci, F., J. R. Broadbent, C. J. Oberg, and D. J. McMahon. 2015a. Growth and gas production of a novel obligatory heterofermentative Cheddar cheese nonstarter lactobacilli species on ribose and galactose. J. Dairy Sci. 98(6), 3645-3654. http://dx.doi.org/10.3168/jds.2014-9293.

Ortakci, F. J., J. R. Broadbent, C. J Oberg, and D. J. McMahon. 2015b. Late blowing of Cheddar cheese induced by accelerated ripening and ribose and galactose supplementation in presence of a novel obligatory heterofermentative nonstarter lactobacilli species. J. Dairy Sci. 98, 7460-7472. https://doi.org/10.3168/jds.2015-9468.

Ortakci, F. J., J. R. Broadbent, C. J. Oberg, and D. J. McMahon. 2015c. Growth and gas formation by *Lactobacillus wasatchensis*, a novel obligatory heterofermentative nonstarter lactic acid bacterium, in a Cheddar-style cheese made using a *Streptococcus thermophilus* starter. J. Dairy Sci. 98, 7473-7482. https://doi.org/10.3168/jds.2015-9556.

Papademas, P., and T. Bintisis. 2017. Cheddar Cheese. Pages 214-216. Global cheesemaking technology: cheese quality and characteristics. John Wiley & Sons, Inc. Hoboken, NJ.

Somers, E. B., M. E. Johnson and A. C. L. Wong. 2001. Biofilm formation and contamination of cheese by nonstarter lactic acid bacteria in the dairy environment. J. Dairy Sci. 84:1926-1936. https://doi.org/10.3168/jds.S0022-0302(01)74634-6.

Tinson, W., M. F. Ratcliff, A. J. Hillier, and G. R. Jago. 1982. Metabolism of *Streptococcus thermophilus*. 3. Influence on the level of bacterial metabolites in Cheddar cheese. Australian Journal of Dairy Technology 37:17-21.

United States Food and Drug Administration. 2018. Generally recognized as safe (GRAS). Accessed July 14, 2018. http://www.fda.gov/food/ingredientspackaginglabeling/gras/.

Wilkinson, M. G. Acceleration of cheese ripening. In Cheese: Chemistry, Physics and Microbiology. Pp. 523-555.

Williams, A. G., S. E. Withers, and J. M. Banks. (2000). Energy sources of non-starter lactic acid bacteria isolated from Cheddar cheese. Int. Dairy J 10:17-23. https://doi.org/10.1016/S0958-6946(00)00027-3.

Wu, Q., C. K. W. Cheung, and N. P. Shah. 2015. Towards galactose accumulation in dairy foods fermented by conventional starter cultures: challenge and strategies. Trends in Food Sci, Tech. 41: 24-36. http://dx.doi.org/10.1016/j.tifs.2014.08.010.

Zheng, J., S. Wittouck, E. Salvetti, C. M. A. P. Franz, H. M. B. Harris, P. Mattarelli, P. W. O'Toole, B. Pot, P. Vandamme, J. Walter, K. Watanabe, S. Wuyts, G. E. Felis, M. G. Gänzle and S. Lebeer. 2020. A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus Beijerinck* 1901, and union of *Lactobacillaceae* and *Leuconostocaceae.* Int. J. System. Evol. Microbiol. 70(4): doi.org/10.1099/ijsem.0.004107.

APPENDICES

APPENDIX A. PROTECTIVE CULTURE INHIBITION OF **PAUCILACTOBACILLUS WASATCHENSIS** WDC04 PLATE COUNTS STATISTICS

Table 9. Comparison of plate counts for *Paucilactobacillus wasatchensis* WDC04 grown with protective culture (indicated in level column by protective culture name) or in pure culture (indicated in level column *Paucilactobacillus wasatchensis* WDC04) was statistically measured using Tukey HSD for Days 0 (A), 4 (B), 8 (C) and 12 (D). Levels with the same letter are not statistically different. Significance was determined at $p < 0.05$.

A.

Level		Mean (Log)
UW4	A	5.36
7995	A	5.25
23F	A	5.25
FBB61	A	5.20
WDC04	A	5.17

B.

Level			Mean (Log)
WDC04	A		9.27
7995	B		8.51
UW4	B	C	8.44
FBB61	B	C	8.25
23F		C	8.17

C.

Level		Mean (Log)
WDC04	A	9.50
FBB61	B	7.91
23F	B	7.83
UW4	B	7.78
7995	B	7.39

D.

Level			Mean(Log)
WDC04	A		9.08
FBB61	B		8.00
UW4	B	C	7.72
7995		C	7.66
23F		C	7.64

APPENDIX B. GAL+/LAC- PROTECTIVE CULTURE EXPERIMENTS

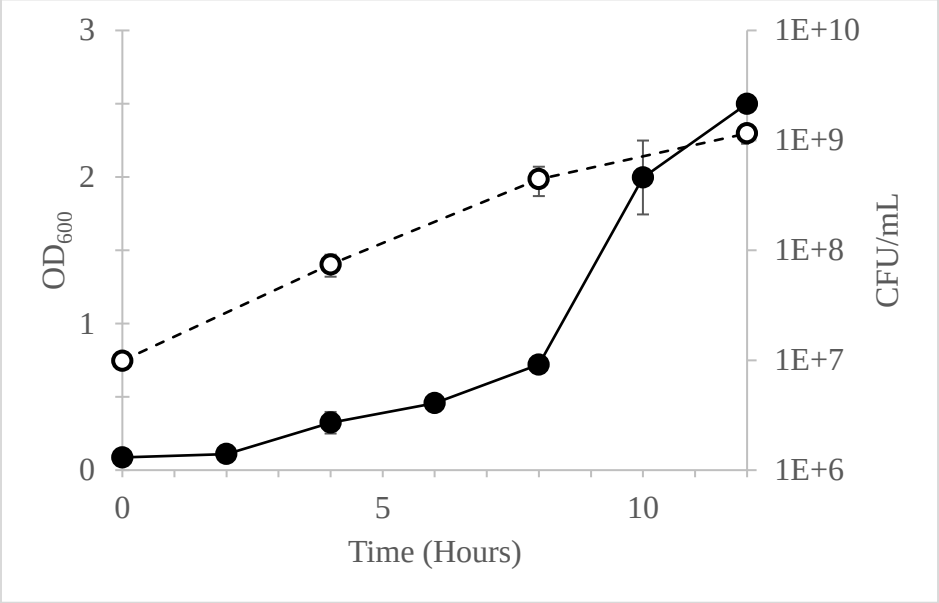

Figure 20. Growth of *Pediococcus pentosaceous* FBB61 in MRS+Gal broth was measured using CFU/mL (dashed line, open marker) and optical density (solid line, closed marker). Error bars=SE, n=3.

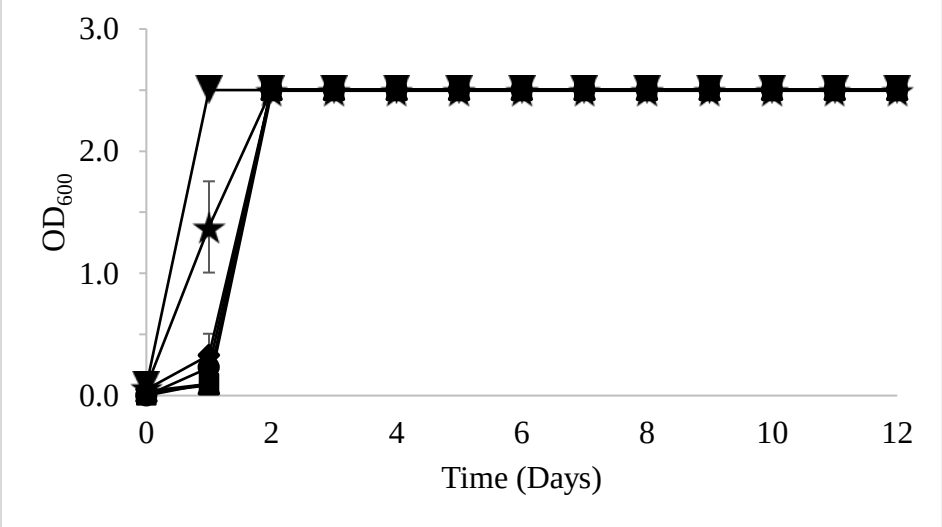

Figure 21. Growth of *Pediococcus pentosaceous* FBB61 was observed alone at an initial concentration of 105 CFU/mL (●) or with Paucilactobacillus wasatchensis WDC04 (initially added at 105 CFU/mL) at initial concentrations of 103 (■), 104 (▲), 105 (♦), 106 (★), or 107 (▼) CFU/mL protective culture using OD600. Error=SE. Control n=2, treatment n=3.

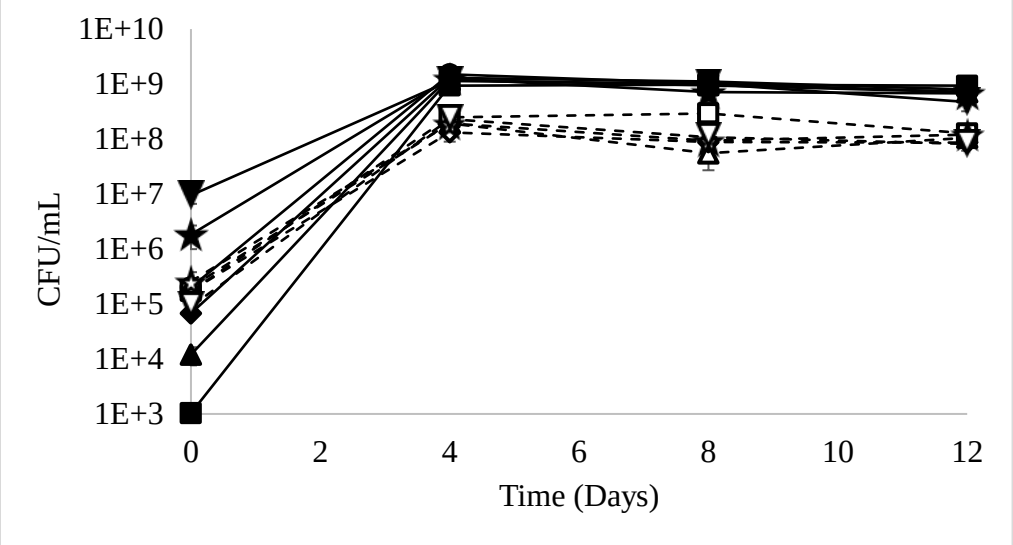

Figure 22. Growth of *Pediococcus pentosaceous* FBB61 (solid line, closed symbol) was observed alone at an initial concentration of 10^5 CFU/mL (●) or with *Paucilactobacillus wasatchensis* WDC04 (initially added at 10^5 CFU/mL; dashed line, open symbol) at initial concentrations of 10^3 (■), 10^4 (▲), 10^5 (♦), 10^6 (★), or 10^7 (▼) CFU/mL. Protective culture was measured in CFU/mL on MRS+R spread plates. Error=SE. Control n=2, treatment n=3.

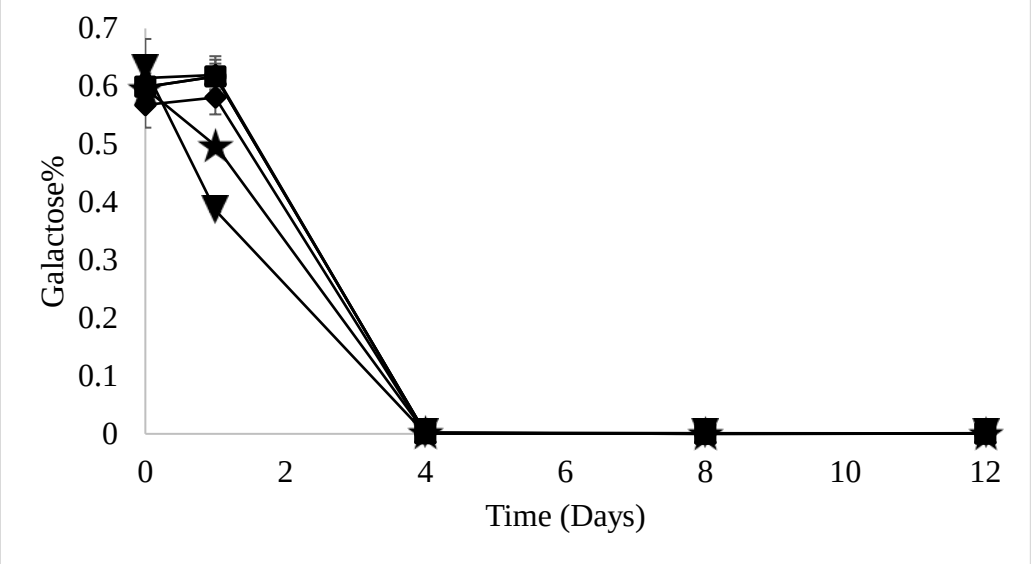

Figure 23. Galactose utilization by *Pediococcus pentosaceous* FBB61 was observed alone at an initial concentration of 105 CFU/mL (●) or with Paucilactobacillus wasatchensis WDC04 (initially added at 105 CFU/mL) at initial cxoncentraions of 103 (■), 104 (▲), 105 (♦), 106 (★), or 107 (▼) CFU/mL was measured by percent galactose using an enzymatic method at 0, 1, 4, 8, and 12 d. Error=SE. Control n=2, treatment n=3.